

Reagan flirts with pie in the sky

The United States is drifting into planning star wars without understanding the costs and dangers: the President needs better advice.

HAD it not been for the invasion of Grenada and the continuing preoccupation of the United States with the predicament of its marines in Beirut, President Reagan's most difficult decision last week would have been to respond to a report recommending that the United States should begin to develop a space-based system of defence against ballistic missiles. The report, devised by a senior interagency group following the President's notorious "star wars" speech in March, was the agenda for a meeting early last week between the President and the Joint Chiefs of Staff. In the event, the meeting was eclipsed by the rapid pace of events elsewhere. But President Reagan's decision on whether to adopt the report's recommendations may be far more fateful in the long run than either of the international crises now distracting him. For if the United States does indeed start work on the new defensive system, nuclear competition between the superpowers will enter a new and dangerous phase from which it will be difficult if not impossible to draw back.

The belief that futuristic technologies could enable the United States to build an almost perfect shield against ballistic missiles, and thereby liberate the world from the insecurities of the existing philosophy of Mutual Assured Destruction, is very much the President's own. His espousal of the idea in his March speech took by surprise many of his closest advisers, and virtually the entire Department of Defense. Until March, defending the "high frontier" had been the harmless hobby of a little-known pressure group led by a retired general and of a minority of physicists with unconventional views, notably Edward Teller. Even after the speech, neither the scientific community nor the general public was disposed to take the idea very seriously, as the spontaneous adoption of the derogatory title "star wars" made clear. The President, it was said, had never been accused of being a technical man. After a polite display of interest, his advisers — scientific, military and diplomatic — would explain to him that such a system could not be made to work sufficiently well and that any attempt to build it would shatter the 1972 Anti-Ballistic Missile Treaty, which although unrattified remains one of the few achievements of arms control negotiations.

It is no longer possible to be so optimistic. Details of the report now before the President, comprehensively leaked in *Aviation Week and Space Technology* (see *Nature* 27 October, p.752), suggest that the groups set up to investigate the feasibility of star wars have taken an extraordinarily starry-eyed view of what can be achieved technically and of the probable Soviet response to such an initiative. The President is being told that rapid advances in directed-energy weapons, precision sensors and information processing have, for the first time, made it possible to conceive of an array of weapons capable of destroying a large, but unspecified, proportion of ballistic missiles shortly after they had been launched against the United States. It is conceded that some missiles would leak through even the most sophisticated defensive shield — but that does not matter: the system would diminish the confidence with which the Soviet Union could consider a disarming first strike against missile silos, and it would force the Soviet Union to choose between a softened stance on arms control or the development of costly countermeasures that would bleed its economy in an unequal competition with superior US technology.

The snag with this rosy scenario is that the Soviet Union does not share the patriotic faith of President Reagan's advisers in the

intrinsic superiority of American technology. Just as defensive measures considered in the late 1960s prompted the development of multiple independent warheads, so the development of a star wars system will compel the Soviet Union to develop new offensive technologies to baffle the defence. Contrary to the advice President Reagan has been given, such measures are almost always simpler and cheaper than the defences they are designed to overwhelm. Missiles can be hardened and thousands of decoys launched. Since the defensive system could not be tested realistically in advance, the United States would not know until too late whether its technology was ingenious enough. A small change in technology, or a well-kept secret, could invalidate an entire defensive array. Consider how the use of precision-guided munitions appeared to spell the epitaph of the tank and the bomber in the 1973 Middle East war: by 1982 in Lebanon, the once invulnerable missile system behind which the Syrian army confidently sheltered had been rendered virtually useless.

Such ebbs and flows of strategic advantage have been the product of advances in military technology since before the English longbow tipped the scales in the battle of Crécy. The scale and cost of the system now being recommended to President Reagan are, however, without historical precedent. What is envisaged is a collection of literally hundreds of satellites mounting enough weapons to destroy 1,000 re-entry vehicles. The advisory group's official estimate that deployment of the first tier of the system by the year 2000 will cost some £95,000 million is probably conservative. President Reagan should be told that to invest such huge sums in a system that can never be tested is, to say the least, an historic gamble.

The problem is that the President does not appear to be receiving the balanced advice he has a right to expect from the groups set up to advise him. The interagency study leaked in the press, and now awaiting a decision, appears to have brushed aside much of the sober criticism directed at the "star wars" notion by many experts hitherto part of the defence establishment. Sceptical voices have been deliberately excluded from the panels brought together to consider the consequences of going ahead with the President's plan. They argue not only that a star wars system will become an ineffectual Maginot Line in space, but that it could precipitate war as each side tries to deploy space mines alongside the satellites of the other.

Of all the presidents in recent years, Ronald Reagan has depended most on the advice of the experts around him. It would be a tragedy for the United States and the world if he were persuaded to take a dangerous new step in the nuclear arms race without a clear understanding of the risks involved. □

Agriculture unloved

British critics of agricultural research should stop sniping at their research council.

AGRICULTURAL research seems everywhere to have become the most contentious way of spending public money on science. In the United States, the wayward determination of Congress to keep on dishing out subventions for the land-grant colleges (not all of which have the intellectual standing of the University of California at Berkeley or Cornell University) when the most urgent need



is for support for untied projects is fast becoming a test of the administration's capacity to spend tax revenues wisely. In Britain, the Agricultural Research Council has been the standard whipping-boy of the endless inquiries into British administration of research at least since the late 1960s, when the then government's chief scientific adviser (Sir Solly, now Lord, Zuckerman) took to using allegations of the council's inefficiency as the first step in an argument for sweeping away the whole of the research council system.

Now, the hapless council is being got at from all directions (see page 4) — the Advisory Board for the Research Councils cut its budget a year ago, support from the agricultural departments of the government is declining, the House of Commons Select Committee on Agriculture has been asking for radical change, the Joint Consultative Organization for Research and Development in Agriculture and Food barely conceals its antipathy from the council and all its works and the council has been forced to change its name, grafting on the word "food" in weak-kneed obeisance to the demand that it should explicitly acknowledge that its ultimate beneficiaries include food manufacturers as well as farmers.

What has gone wrong? Part of the trouble is the council's own past doing. It began life (in 1931) when universities as such had no great competence in research, and slipped into the habit of setting up research institutes (many of them originally closely linked with universities, as some still are) to handle specific problems as these were identified. The model was a distinguished one — the Rothamsted Experimental Station which, in the nineteenth century had won an outstanding reputation for the improvement of agricultural productivity. Half a century ago, it would have seemed natural enough that the council should be setting up comparable institutes to look after plant breeding (consistently successful) and animal health (less of a success). In more recent decades, the council has been less careful. Do farmers have a problem with weeds? Then let there be a Weed Research Organization.

The result, for the past fifteen years or so, is that far too great a proportion of the council's budget has been committed to its internal operations, and too little (£3.3 million out of more than £90 million in 1981–82) on research projects not already on the agendas of the various research institutes. It is therefore good news that the strategic plan to be considered by the council in December is to create more room within the budget for spending of this kind. The danger, as things are, is that the pattern of British agricultural research will be overwhelmingly determined by the interests of particular institutes, much as the pattern in the United States is formed by the vested interests of those universities that happen, nearly a century ago, to have qualified as land-grant colleges.

The defect of most of the proposals for changing the administration of agricultural research in Britain now widely in circulation is that they would reinforce that danger. The general cry is that research should be harnessed more directly to the needs of its ultimate beneficiaries, British farmers, fishermen and food manufacturers. As it happens, two quite separate organizations — the Ministry of Agriculture, Fisheries and Food and the Agricultural Development Advisory Service (Britain's own extension service) — ostensibly exist already to cater for many of those needs, but neither of them is equipped to carry out the kinds of research programmes on which future innovations must be based. Genetic manipulation (and the plant physiology on which its applications must be based) and nutrition (human as well as animal) are among the more obvious fields at risk from too slavish a concern for immediate benefit. Moreover, it is hard to see how any other organization than the research council could do such jobs.

Administratively, the most obvious difficulty is that there is no mechanism for carrying out a sensitive analysis of who at present does what with what objectives, and for telling whether the pattern of effort is deficient. To be sure, the agriculture ministry (which ultimately pays the bills) should be doing this, but its chief scientists have never had the clout to tackle such problems — and have even less now that what was once intended to be an influential post has been split into two and downgraded in the civil service

hierarchy. Yet the need for an overall understanding of what is happening in agricultural research is so urgent, and the prospect of it emerging is so slender, that the embattled research council should consider seriously whether to take on this function with its own resources. Subversive as that would seem to the government that pays the bills, the council has little to lose and everything to gain from a serious attempt to ensure that the present strategy of agricultural research is properly understood and that a better strategy is hammered out for the future. The worst that could happen (abolition) is what the council's most fierce critics want. The riposte that much good has been done in the past is not an effective defence — an imaginative prospectus might be. □

Opportunity for Mr Blix

The International Atomic Energy Agency is doing well. Could its influence be enlarged?

RUCTIONS though there may be at Geneva about the future of arms control negotiations, the administration of the international safeguards required by the Non-Proliferation Treaty seems to be as smooth as anybody could expect. That is one of the more obvious inferences to be drawn from last month's meeting of the general conference at Vienna of the International Atomic Energy Agency. The days have gone when the agency had too small a budget to cover the cost of the inspections required of it — and could not, in any case, recruit the trained people needed to undertake this exacting work. Helped no doubt by the slower than expected pace of growth in the nuclear industry, the agency is somehow able to convey to its members a sense of confidence that non-nuclear powers that have signed the treaty, and which have by so doing agreed to accept full safeguards, are not using their nuclear industries for illicit purposes on the side. It is, however, worth remembering that in the last resort what matters is not so much the technical adequacy of the inspection procedures as the readiness of governments to accept the need for them. In this sense, this year's meeting at Vienna seems to have been helped along by the decision of the Soviet Union a few months ago that some of its nuclear plants should be voluntarily opened to inspection, as are plants in other nuclear powers such as Britain and France.

So far so good — but what about the governments that have hitherto declined to sign the Non-Proliferation Treaty? Less than two years from now, the rules of the treaty will require that there should be another conference to review the way the treaty is working. The contentious issues in 1985 will be what they were three years ago — why have not the nuclear powers done more to implement their promise to negotiate agreements on strategic arms? And there will persist the nagging doubt that those governments ostentatiously unwilling to sign the treaty while remaining members of the international agency (such as Argentina, Israel and South Africa) may be making nuclear weapons behind everybody's backs. (This year's general conference adopted a resolution drawing attention to the problem of South Africa, urging other states not to help its government make nuclear explosives, but said nothing about the other black sheep.)

Dr Hans Blix, the secretary-general of the agency for the past two successful years, could usefully devote some of his fund of energy and imagination to this problem against the coming review of the Non-Proliferation Treaty. For while there is nothing that the Vienna Agency could do to bridge the kind of gap that has grown up between the superpowers at Geneva, there is much that could be done by talk and example to demonstrate to governments remaining outside the treaty that they have more to lose than to gain by their obduracy. At the very least, they will be the less able to add their voices to the chorus of complaint there will be in 1985 if nothing is done about strategic arms control. So long as the agency remains one of the few durable international monitors of good conduct, there must be hope that its influence will grow. □

US space science

Space agency pushing for a bigger budget

Washington

THE financial cloud that has recently darkened space science in US universities is showing the first hint of a silver lining. The National Aeronautics and Space Administration (NASA) has told university researchers that its 1984 budget submission to the White House includes a strong plea for extra funds to help beleaguered university centres. If approved, NASA's rescue operation will include a special fund to modernize instruments, a new graduate fellowship programme for space science and a big increase in the amount of money available for analysing data from space missions.

In the view of most university space scientists, NASA's action is long overdue: a newly completed report drawn up by a joint NASA/university committee paints a startling picture of university departments unable to buy up-to-date equipment, short of graduate students and frustrated by the dearth of flight opportunities now that NASA's space science programme is focused on long-lived observatories and planetary orbiters. The committee, co-chaired by NASA's chief scientist, Dr Frank McDonald, calls for immediate action to avert a crisis in university morale.

According to the group's report, NASA's relationship with the universities has undergone a profound change since the golden age of the 1960s. Then, the agency's space science programme involved four or five missions a year. NASA gave multidisciplinary research grants to more than 40 universities and supported construction of 37 space science buildings or additions. It also paid for a national programme of fellowships for graduate students; by the end of the decade, more than 5,300 students had received three-year awards.

Today, the launch rate of scientific satellites has dropped to one or two a year and the graduate fellowship programme and the university grants have stopped. The budget of NASA's Office of Space Science and Applications (OSSA), measured in constant dollars, has decreased from a peak of \$1,630 million in 1964 to \$950 million in 1984. Because opportunities to fly experiments have at the same time decreased, 'drastic reductions' have been forced in many university space research groups.

Urging "extraordinary steps" to deal with the problem of ageing equipment, the report recommends that \$11 million a year be earmarked for five years from 1985. The money would be spent on commercial laboratory equipment such as oscilloscopes and spectral analysers, small and medium size computers and major facilities such as

micro-ion probes, gas analysers and large fast computers in the Cray class.

The report also recommends the immediate reintroduction of a NASA graduate research fellowship programme, beginning in 1985 with 50 fellowships and expanding to a level of 200 a year, to encourage bright undergraduates to pursue graduate work in sciences. Stipends would cover the full cost of tuition and include a living allowance of at least \$13,000 a year.

Finally, NASA wants a larger budget for data analysis and ground control. The report points out that the fall in the number of new missions has been partly offset by the increased longevity of spacecraft; at present, 14 active satellites are returning valuable new space science data, but funds to process the information have been scarce.

In 1983, the data analysis and mission operation part of the OSSA budget amounted to \$155 million, about 15 per cent of the total. The report proposes an increase of about \$20 million a year, with most of the extra money going to university groups. Some \$10 million would be allocated to Solar System exploration data, \$4 million to astrophysics and \$6 million to environmental observations.

If accepted by the Office of Management and Budget — and later by Congress —

NASA's package of spending increases will be a magnificent tonic for university space scientists. But the package will not deal with the problem that most worries university researchers, their diminishing access to space flights. In a strongly-worded letter to the committee, members of the University of Washington's McDonnell Center for the Space Sciences that unless new missions were conceived and flown, whole disciplines — such as X-ray astronomy — could die.

Access to the shuttle has been the biggest disappointment. The Washington group claims that, when it wants to, NASA can help to mount experiments with remarkable speed. When the agency discovered last year that plastics were becoming dangerously eroded in space, it commissioned an emergency experiment, and the group was able to analyse the results only four months after being notified of the problem. But the group has now been told that it will be two years before it can fly another experiment.

In another letter to the committee, Dr Peter Banks, director of the Space Telecommunications and Radioscience Laboratory at Stanford University, predicts that the situation would not improve even when the Spacelab series is under way. The long delays and the emphasis on large-scale management and engineering projects put Spacelab experiments beyond the reach of most university groups. In the long run, he warned, NASA would dominate the development of space science facilities, with universities playing an ever diminishing part.

Peter David

Biotechnology

Pessimism about patent rights

Washington

BOTH patents and trade secrets may be "anachronisms", of little value in the fast-paced biotechnology industry, J. Leslie Glick, president of Genex, told a meeting of patent lawyers last week. Glick and several other speakers at the meeting, sponsored by the American Type Culture Collection (ATCC), said that traditional protection of intellectual property suffers from a number of failings when applied to biotechnology.

Inevitably, Glick said, there will be several solutions of a given problem in high-technology fields, thus providing competition against which patents or registration as trade secrets cannot protect. The typical three-year lag between the filing and issuance of a patent also limits the usefulness of patent protection.

The peculiar requirements that go with the award of biotechnology patents further tend to limit the scope of protection accorded. The legal requirement that a patent should fully disclose an invention, so that a person "skilled in the art" can reproduce it, must be met in the case of most

biotechnology patents by depositing a sample of a novel organism in a public repository such as ATCC. These deposits, not required for patents in other areas, can "markedly reduce the research and development expenses" of a competitor seeking to improve on the invention or to evade the scope of the patented invention, according to Dale Hoscheit, a Washington patent lawyer.

Although hybridomas have been licensed as trade secrets they too present unusual problems. Limitations on free publication or on the exchange of materials among researchers is the most obvious drawback. Then state courts often require proof of even physical security before finding in favour of the owner of a trade secret who accuses a competitor of unlawfully taking his property — a condition not easily met when university laboratories are involved. And "reverse engineering", the reconstruction of a process from a knowledge of its end-product, although not an immediate danger with hybridomas, is always a risk with trade secrets. **Stephen Budiansky**

Agricultural research

UK council forced to trim sails

THE British Agricultural Research Council (ARC) is pressing ahead with a radical restructuring of its support for research, apparently unabashed by its rough treatment last year by the Advisory Board for the Research Councils. The council, soon to be known as the Agricultural and Food Research Council, is increasing support for food research at the expense of most other areas and plans to increase by 50 per cent its support for research undertaken in universities. University research will in future take up 15 per cent of ARC's share of the science budget.

When the advisory board's "Forward Look" was published a year ago, ARC's secretary, Dr Ralph Riley, protested at the proposed decline in ARC's share of the science budget. But ARC is now planning for next year on the assumption that its real income will be even less than the advisory board recommended, mainly because of a Treasury decision to restrict the money available for pay increases to 3 per cent rather than the expected 5 per cent.

Although ARC will not formally know until the end of the year how much cash it will have during 1984-85, it is now assuming that the worst will happen. It expects to receive only £45.9 million from the science budget, significantly less than in the current year, and is reconciled to less commissioned research from the Ministry of Agriculture, Fisheries and Food than had been hoped for.

ARC's internal allocation of funds to its institutes will not be finally decided until a strategic plan is completed in December. Meanwhile, however, most ARC institutes have been asked to submit proposals for large savings, including redundancies where necessary. It has already been decided that 13 members of staff will lose their jobs at Rothamsted Experimental Station.

Most of the planned reductions are in animal disease, fruit and arable crop programmes. In percentage terms, the largest cuts will be made at the Institute for Research on Animal Diseases near Newbury and at the plant physiology Letcombe Laboratory near Oxford. It seems likely that there will have to be major changes in the work of some smaller institutes such as the Letcombe Laboratory and the nearby Weed Research Organization.

Earlier this year, ARC estimated that if the planned cuts went ahead, 600 posts would be lost over three years but that roughly half of their holders might be redeployed. Scientific staff would account for two-thirds of the redundancies.

The only institutes that will receive a large cash increase next year are the Food Research Institute at Norwich and the Meat Research Institute near Bristol. The shift towards food research was recommended in a report last year by the

Advisory Council on Applied Research and Development, and ARC has now obligingly formally created a Food Division.

Some policy makers feel that, with food surpluses accumulating to an embarrassing degree in Europe, there is a greater need for research on how to use food than how to produce it. Others, such as Mr J. Fryer, director of the Weed Research Organization, say this is a short-sighted view, and that there will be more rather than less need for mainstream agricultural research during the rest of the century.

Fryer is particularly concerned that

political pressures may reduce Britain's ability to carry out research relevant to the needs of developing countries. He is worried that ARC might be forced to make decisions whose effects will span decades in response to short-term considerations of expediency.

In ARC's draft annual report for 1982-83, Dr Riley says "... it is important to hold the course that has been so effective ... namely that of providing a wide range of scientific information from which an array of practical technology may be drawn".

Many institute directors are keeping their fingers crossed that Dr Riley will have the clout to persuade the council's paymasters of just that principle.

Tim Beardsley

Animal welfare

Berkeley campus in dog-house

Washington

THE University of California at Berkeley, which for years has been criticized for inadequate care of laboratory animals, is now facing opposition from animal rights groups to its plans to construct improved laboratory facilities.

At an unusual meeting with the regents of the university last week, three animal rights groups (Fund for Animals, Californians for Responsible Research and a group calling itself Buddhists Concerned for Animals) pressed their case, repeating now-familiar charges of substandard animal care at Berkeley. Animal rights activists earlier this year succeeded in persuading the state legislature not to approve construction funds for a new life sciences building until the university demonstrates that conditions have improved.

The legislature is specifically requiring Berkeley to meet the strict standards set down by the American Association for the Accreditation of Laboratory Animal Care (AAALAC). A request for construction funds is expected from the university next year.

Berkeley's chancellor, Ira Heyman, last week responded to the animal rights groups' charges by saying "we cannot meet our goals for completely up-to-date and effective animal care without new buildings". The major biological laboratories at Berkeley are old, one of them more than 50 years old. Heyman said that "it is impractical or impossible" to bring these older buildings up to modern standards. And he accused the groups of attempting to portray Berkeley in the worst possible light by presenting specific past problems as typical of the campus as a whole.

Mark Giannelli, science adviser to the Fund for Animals, said he does not oppose construction of the new laboratory (although the two other groups that spoke before the regents last week are preparing a lawsuit against the university, demanding

that an environmental impact statement on the new buildings be filed) but added that the "primary problem" in animal care at Berkeley is one of "attitude", not facilities.

Inspection reports of the US Department of Agriculture (USDA) (which enforces the Animal Welfare Act), AAALAC, and several internal Berkeley committees tend to support this view. An AAALAC inspection requested by Berkeley in 1982 cited nine general deficiencies that would have to be corrected before full accreditation could be granted. Of these, only one — inadequate ventilation and temperature controls — involved facilities directly. A faculty task force concluded last February that it was not at all clear "whether a new facility will solve all the animal problems of the Berkeley campus".

The USDA inspection reports provide more details in a litany of complaints of housekeeping and sanitation deficiencies. Clogged drains, feed mixed with excrement, protruding sheet metal edges and wire in cages, insect and wild rodent infestations and a lack of trained employees are cited in report after report. USDA inspectors have filed several alleged violations of the Animal Welfare Act with USDA's office of the general counsel. Sources close to the USDA investigation of these charges say that the agency will seek civil penalties against Berkeley.

The Berkeley campus has recently begun to correct many long-standing problems. Half a million dollars has been allocated since July 1983 for major renovations, mainly improving temperature control and installing new cage washing facilities. Two additional campus veterinarians have been hired, and a training course for animal caretakers set up. And, in response to the legislature's requirement, Berkeley has invited AAALAC to reinspect the campus "in a few months", the chancellor said.

Stephen Budiansky

Biotechnology regulation

Rules for freed organisms planned

Cambridge, Mass.

ENVIRONMENTAL regulation of biotechnology has come one step closer. Attorneys in the Office of General Counsel at the Environmental Protection Agency (EPA) have advised Donald Clay, the acting assistant administrator for pesticides and toxic substances, that the agency has "inherent statutory authority" over release of genetically-engineered organisms into the environment.

The first of two opinions sent to Clay states that recombinant DNA molecules can be considered "new chemical substances" whose commercial introduction should be controlled under the federal Toxic Substances Control Act (TOSCA). The second declares that the release of ice-nucleation bacteria that cause frost sensitivity on plants whose ice-nucleation genes were deleted by recombinant DNA techniques comes under the jurisdiction of the federal Insecticide, Fungicide and Rodenticide Act (FIFRA).

Clay is expected in both cases to accept the advice. Indeed, EPA considers regulation of recombinant organisms to be a natural extension of its influence over new chemicals and pesticides, with no further legislative or regulatory action required. EPA intends to issue a policy statement on regulation of recombinant organisms under TOSCA next spring, setting out in general terms the role it intends to play, addressing some of the technically difficult issues and defining the conditions under which recombinant DNA is to be considered a new chemical.

In due course, EPA will ask for public comments and advice on its policy statement. After further review, EPA will issue more formal guidelines for companies to follow before producing organisms whose genes have been modified by recombinant DNA techniques.

The rationale for controlling recombinant organisms under TOSCA is based on the statutory definition of a new chemical. Risk analysis in conformity with TOSCA's guidelines would obviously not be carried out on the DNA molecules themselves but on their bioactive form, as components of microbial, plant or animal genomes.

TOSCA specifically requires companies to provide pre-manufacture notification before the commercial introduction of a new chemical. EPA then has 90 days to review the proposal. If EPA takes no action, the company may go ahead with manufacture. EPA may otherwise request more information, or it can ban manufacture of the new chemical outright. Excluded from TOSCA's purview are any chemicals regulated under FIFRA or the Food, Drug and Cosmetic Act. Moreover, TOSCA does not allow interference in research and testing on new chemicals.

Officials at EPA admit that their new in-

terpretation of recombinant DNA is likely to stir up controversy in the biotechnology industry; they would not be at all surprised if someone decides to sue over this extension of their authority. Nonetheless, EPA suspects that most companies will be willing to go along with these initiatives, since regulation of some kind appears inevitable. No one wants to see the passage of additional legislation — which would almost certainly be more restrictive.

EPA also believes that the regulation of pest-control organisms under FIFRA is likely to be comparatively clear-cut, since the agency already oversees release of natural biological control agents. The Office of General Counsel's opinion on release of the modified ice-nucleation bacteria may well set a precedent for considering as "pesticides" any genetically modified organisms designed to control, displace or suppress pests. The FIFRA requirements include pre-market registration of pesticides that are to be tested on a plot larger than 10 acres, and application for a licence before commercial manufacture of any new pesticide.

The Office of Pesticide Programs (OPP)

will probably not handle genetically-engineered pest control agents in a manner significantly different from its approach to natural agents. Since no one has submitted a recombinant organism for registration or licensing under FIFRA, the office is still informally considering whether and how genetically-engineered agents might be treated differently from the natural ones. OPP is consulting a variety of experts and the National Institutes of Health's Recombinant DNA Advisory Committee to determine whether there is any basis for regulating more stringently the release of recombinant organisms.

OPP will at least want to know about the parent organism's genetics, physiology and ecology, as well as the ways and extent to which it has been modified. It is also considering eliminating the 10-acre exclusion, EPA's attorneys having suggested that OPP use its authority to require an experimental use permit for any field testing of an engineered agent, on whatever scale.

A draft proposal of new requirements for pesticide registration under FIFRA is now passing the numerous hurdles within the agency that precede approval. EPA hopes to issue them under Administrator William Ruckleshaus's signature in January or February. **Christopher Earl**

Windscale

Increased cancer incidence alleged

BRITISH Nuclear Fuels Ltd (BNFL) has reacted angrily to allegations in a television documentary broadcast in Britain this week that cancer incidence among children near its waste reprocessing plant at Sellafield (formerly Windscale) is several times higher than the national average.

The documentary, *Windscale — the Nuclear Laundry*, was produced for Yorkshire Television by Mr James Cutler. According to Cutler, cancer cases among children under the age of 10 in the village of Seascale, one mile from the plant, are 10 times more frequent than in Britain as a whole. Cutler says that excess cancer cases occurring over more than 25 years are clustered along the Cumbrian coast near Windscale. He claims that the results were described as "very disturbing" by BNFL's independent assessor.

The Yorkshire team obtained analyses of radionuclides in household dust near the plant from Dr Philip Day of the University of Manchester and Professor Edward Radford of the University of Pittsburgh. Both found radionuclides in the samples with isotopic ratios corresponding to those in

known discharges.

The National Radiological Protection Board has never sampled household dust in Cumbria, although it has monitored airborne activity out of doors and finds it well within acceptable limits. The board has seen results of some of the dust analyses and estimates dose from this route to be small. It is conducting a major epidemiological survey of cancer incidence in Cumbria, but says the results will not be available for some time.

BNFL points out that there will always be regional variations in cancer incidence, and that no significance can be attached to Cutler's figures. Even by Cutler's activity data, it says, annual dose would be less than one per cent of safety limits. BNFL has complained to the Independent Broadcasting Authority about the research techniques used by the Yorkshire Television team, which allegedly included misuse of confidential medical files, and has received apologies from the authority. BNFL is also angry that Cutler made no use of the company's own epidemiological studies on its workforce and said on Monday this week that it would decide whether to make a formal complaint about, "one-sided advance publicity" only after finding out whether the television company would allow it as promised to reply "within the programme" to any criticisms made of it. BNFL's contribution was due to be filmed on Tuesday. **Tim Beardsley**

Correction

Nature is happy to report that Jerrold R. Zacharias, emeritus professor of physics at Massachusetts Institute of Technology, is very much alive, contrary to the implication in the issue of 29 September, p.348.

Job discrimination in Italy

Brussels supports poor cousins

FOREIGNERS employed by the Italian national research council (CNR) have obtained strong backing from the European Commission in Brussels in their struggle for the same rights as Italian scientists, job security in particular.

For years now, the 18 scientists (the number was much larger, but has dwindled as the conflict has dragged on) have been switched from one short-term contract to another, although Italian colleagues of the same standing were granted tenure and improvements in salaries and conditions. In 1982 the contracts of all 18, scattered throughout many disciplines and throughout Italy, were terminated by CNR, but they were then re-employed as consultants by the institutes at which they worked but without pension and other rights. In January this year, the 18 won further two-year contracts, after heavy pressure on CNR, and the institution of legal proceedings (which may take three years to come to court) on their behalf.

That is not enough, says the Commission's director-general for employment and social affairs, Jean Degimbe, in a letter sent on 22 August to the Italian ambassador in Brussels. In the letter, released by the 18, Degimbe says that the failure to give permanent jobs to these research workers "constitutes discrimination in working conditions" compared with their Italian colleagues. The letter refers to the unequal conditions of pay and status and says that their employment is "precarious" in contrast with the stability enjoyed by their Italian colleagues.

The letter charges that the CNR action is contrary to the European Economic Community (EEC) treaty and in particular to paragraph 4 of article 7 of Regulation EEC/1612/68, which decrees that contracts or rules of employment that discriminate against citizens of other member states "shall be null and void". Degimbe then requests, within two months, a copy of the measures adopted by the Italian Government in order to conform with the directives of the Community in this matter.

So are Italian legislators now shaking in their shoes? Probably not, for in another letter obtained by the 18, an Italian official in Brussels forwards Degimbe's letter to Rome with the remarks that "as far as we are able to determine . . . the Commission does not think the time is ripe for a comprehensive solution of the problem, given the present difficult economic conditions, which encourage protectionism and isolationism . . . The Commission therefore limits itself to following up the protests and claims received, selecting those that make suitable test cases to ease the path towards a real solution".

However, the official adds, the Commission is close to making a proposal for a

directive on transnational employment. Such a step would bring the issue to the political level of the Council of Ministers. The official also points out that CNR cannot evade its responsibilities by claiming that CNR scientists, as civil servants, are immune from the application of the anti-discrimination directives because of the way in which the Treaty of Rome excludes posts in public administration. The Commission takes the view, says the Italian official, that this qualification applies only to posts involved in the "exercise of civil power".

What will happen now is far from clear. Brussels is awaiting a reply, and is said to be considering taking Italy to the European Court if no satisfactory solution is forth-

coming. But Italy can delay action pending the outcome of the case laid by the 18 against CNR under Italian national law. A spokesman for the 18, ex-Belgian (now Italian) Julien Houben, a biophysicist at Pisa, is not concerned, however: he feels that the Brussels letter will strengthen their case in Italy and pave the way for a political solution.

At CNR, officials claim they are caught in a legal dilemma: its *parastato* (civil service) statutes forbid it to employ foreigners, so it cannot hope to obey both Italian and EEC regulations. The answer thus seems to lie with the Italian Government, which no doubt is why the 18 are now seeking interviews with the Minister of Research and with the Prime Minister, socialist Bettino Craxi. The 18 hope to know their future prospects "within 15 days" said a spokesman on Monday.

Robert Walgate

US research support

Academics agree on fair play

Washington

PORK-barrel politics may soon be a thing of the past in the competition for federal research funds if leading research universities in North America adhere to their recent gentleman's agreement. At its annual meeting in Los Angeles last week, the Association of American Universities (AAU) issued a statement affirming that scientific merit and not political influence should determine the distribution of government money for both scientific projects and the facilities to house them.

By issuing the statement, AAU, which represents the 50 major research universities in the United States and Canada, implicitly reprimanded two of its own members. Many university presidents have been angered by the unconventional congressional manoeuvres used earlier this year by Catholic University in Washington, DC and Columbia University in New York. Both universities employed a Washington-based lobbying firm to talk Congress into supporting projects that would not otherwise have been supported (see *Nature* 20 October, p.659).

The statement does not name the two universities but says that the principle of peer review in scientific funding decisions needs to be preserved and strengthened. It calls on university leaders, scientists and members of Congress to refrain from actions "that would make scientific decisions a test of political influence rather than a judgment on the quality of work to be done". The correct procedure, the statement says, is for Congress to appropriate funds for broad categories of research, and for federal agencies then to make individual decisions on the basis of expert advice.

AAU's views that peer review should be applied to buildings as well as to research programmes marks a defeat for Father

William Byron, president of Catholic University. He had argued before the meeting that only the programmes themselves required peer review. But the statement does recognize the temptations facing many universities suffering from a shortage of facilities needed to support worthwhile research.

Since the early 1970s, AAU claims, there has been virtually no federal government support for the construction and renovation of research facilities. The decade of neglect has hampered American science and placed "great stress" on the process by which the government allocates scientific resources.

Peter David

Cuba elected

CUBA is to "modernize and extend" its nuclear research institute, according to the official press agency, *Prensa Latina*. The institute "will be allocated" two new reactors — one of 10 MW and one of "greater power", *Prensa Latina* said. Work is also going forward on the construction of the first Cuban nuclear power station.

The announcement was made to celebrate Cuba's election last month to the governing board of the International Atomic Energy Agency (a board which *Prensa Latina* somewhat unfortunately called the "governors' junta"). Cuba has sought election since 1978, and claims that it would have been elected earlier were it not for "US pressures". In particular, alleged *Prensa Latina*, the United States urged Guatemala to put itself forward as a candidate for the board, which, in view of the "nearly non-existent development of that Central American nation" could well be interpreted as "another US manoeuvre to deprive Cuba of her legitimate right".

Vera Rich

British research councils

More trimming in prospect

CHAIRMEN of British research councils, in their annual reports, have to tread a difficult path between complacency and despair. Sir Hermann Bondi, chairman of the Natural Environment Research Council (NERC) is no exception. In his annual report for the financial year 1982-83, published last week, Sir Hermann paints an encouraging picture of NERC's future prospects against a background of depressing financial news.

Sir Hermann singles out for special mention the work of the British Institutions Reflection Profiling Syndicate, which has produced important results on the border between the Earth's mantle and crust. Another important growth area for the council is the use of satellite data. But, like other research councils, NERC is having to cut back on research in some areas in order to allow for expansion elsewhere. Some 80 posts will be lost during the current year, and next year the target will be a further 120 posts, although many staff will be redeployed.

NERC's biggest financial worry is that research commissioned by government departments has fallen steeply, with the result that total income is now down by 13 per cent on the figure just a few years ago. In order to minimize the effects of this loss, NERC is making energetic efforts to find non-governmental contract research. A Research Marketing Group has been established within the past year for just this purpose, although it has not yet had time to make any significant contribution to NERC's coffers. Dr Keith Harrap, director of the marketing group, says that most of his likely customers are to be found overseas, so NERC is planning to tout its services in Brussels and in Washington (where the World Bank may have work to offer). The marketing group also hopes shortly to secure a major contract to investigate marine pollution in Oman.

Meanwhile, NERC has maintained the value of the research it supports in universities, despite the overall contraction. Acid rain is, of course, now a boom area. Owing to the reductions in universities' recurrent grants, however, the number of applicants for research funds has increased dramatically, with the result that many more of them had to be turned away last year than previously. The council is hoping to hold steady the value of university research in future years, in line with general recommendations made to the research councils two years ago.

On the vexed question of achieving the right balance between university and in-house research, Sir Hermann says: "I do not think that it is possible . . . for us to step into the breach left by the weakness of the other pillar of the dual support system. In fact, I think it is debatable whether . . . we should even attempt to do so."

The projected level funding for university-based research has had to be paid for in NERC's own institutes. The main research areas that have been hit are conservation studies, pest control and, in the geological field, production of a series of six-inches-to-the-mile geological maps. The council also reports that it failed to convince the government of the need for more research on atmospheric carbon dioxide. Two stations of the Institute of Terrestrial Ecology are to be closed, and staff at two laboratories run by the Institute of Oceanographic Sciences may be relocated. One possibility the council is considering is that it might establish a

number of multi-institute sites.

NERC participates in several international research projects, but problems over exchange rates may curtail some of them. British participation in deep sea drilling remains uncertain after the Deep Sea Drilling Project ends this month.

One research area where NERC can be optimistic, however, is the British Antarctic Survey. After the hostilities in the South Atlantic last year, government support for the survey was increased, and new buildings have now been constructed at Halley Station, to replace those being crushed under several years' weight of accumulated snow. NERC expects to be able to maintain, if not increase, opportunities for university scientists wishing to work in the Antarctic.

Tim Beardsley

Dutch science

Ministry plans intervention

The Hague

THE conservative coalition government of the Netherlands seems devoted, like the Thatcher Government in Britain and the Reagan Government in the United States, to less government — but with a single exception: science. Or so it seems to many Dutch scientists, who are now beginning to organize resistance to a measure which they consider will "politicize" the Dutch basic research council ZWO.

The minister of education and science, Mr Wim Deetman believes that the Dfl 200 million (£45-million) a year ZWO has hitherto been too independent, serving the interests of Dutch scientists but not of the economy. In his view it has been too protective, not selective enough. But ZWO director Professor van Lieshout counters that ZWO has been restricted by a statute which explicitly prevents it from carrying out applied research. "We would love to do applied research," he said last week.

Applied research ZWO will now do, according to the minister's draft statute for the organization; and it will merge with its smaller sister (the Dfl 12 million a year STW) for that purpose. Thus far, there is agreement. According to the chairman of the influential Dutch Council for Science Policy (RAVW), Professor van Bueren, "we are quite agreed that science must help the government in health and the economy and so on". But the question, says van Bueren, is "how far should it go". For according to the minister's proposals, political factors would become obtrusive.

At present, ZWO is headed by the president and a 40-strong council chosen by the ministry from a list of nominees put forward by each university and by other groups. In selecting the council, the ministry relies on advice from the Royal Academy of Arts and Sciences (RAAS) which has in the past been "rather influential" according to van Lieshout.

In future, however, the minister's pro-

In future, however, the minister's proposal would do away with the council and RAAS would have no role in selecting ZWO officials. Instead, the minister will directly appoint the 11-strong executive board, which is now chosen by the council without supervision. Moreover, the minister will also appoint the sub-boards — the five committees that are closest to the work-face of science, managing ZWO's various areas of work. Without the council, which met three or four times a year and approved — or disapproved — the ZWO budget as proposed by the board, power in ZWO would be much more centralized, van Bueren believes, and would be centralized in political hands.

All in all, this open disagreement, which will finish only when a final proposal goes to cabinet in the spring of 1984, appears to be one more painful step in a determined government programme to take closer control of the academic establishment in the Netherlands. Thus in the universities, budgets are being cut some 4 per cent a year over the next four years, professorial posts axed by 5 per cent a year (100 will retire next year when the compulsory retirement age is reduced from 70 to 65) and government money will be allocated for the first time according to research "programmes" assessed (somewhat at present) by the ministry.

However Dutch universities, on the admission of their own staff, have long been "the richest in the world", receiving more unearmarked support for research than universities anywhere else. Hitherto, ZWO has provided only some 7-10 per cent of academic research support, the rest coming direct from the ministry to the university, for redistribution locally. Among governments that channel academic research support by more than one route, the Dutch has stood out for the freedom given to universities.

Robert Walgate

Yellow rain

Fresh support for apian origin

YELLOW rain may not be a chemical warfare agent after all. According to four scientists in the geology section of China's Nanjing University, it is a natural phenomenon. In a recently discovered paper published in a Chinese journal, *Kexue Tongbao*, in September 1977, the scientists describe "yellow rain" in the central province of Northern Jiangsu and say that it was probably bee excrement released by the insects on cleansing flights.

The same theory has been put forward by Professor Matthew Meselson of the Department of Biochemistry and Molecular Biology of Harvard University to explain the presence of yellow rain in the South-East Asian countries of Laos and Kampuchea (see *Nature* 303, 3; 1983).

According to the United States State Department, yellow rain is a chemical warfare agent being used by Soviet-backed forces in Laos and Kampuchea to harass and kill refugees and enemy soldiers. The Soviets have consistently denied these allegations.

It is far from clear why the Chinese paper, "A study of the origins and the pollen analysis of 'yellow rain' in Northern Jiangsu" (*Kexue Tongbao* 22, 409-412; 1977) has taken so long to surface. Britain's Chemical Defence Establishment at Porton Down has been aware of its existence for some time but has made no public comment about its contents.

Writing in language used during China's cultural revolution, the scientists, Zhang Zhongying, Chen Yuming, Zhou Shu and Li Min, describe their investigation in Jiangsu in September 1976. According to the authors, the "masses" were concerned about the "yellow rains" which were falling over the countryside and, true to the spirit of the times, scientists were sent to investigate.

The yellow rain is described as being glutinous and falling in showers lasting sometimes as long as ten minutes. On the ground, circular or oval spots were yellow or brownish-yellow in colour, slightly raised and looked like half a soya bean. The spots varied in size from 2 to 6 mm and covered areas varying in size from a few acres to more than 100 acres.

Analysis of 500 spots confirmed that they were composed primarily of pollen grains. Most of the pollen (83 per cent) was from a species of elm, *Ulmus parvifolia*, which flowers in September. Some 11.8 per cent of the pollen was from Gramineae and 3 per cent from Compositae. There were no spores from fern. Yellow rain collected from two districts, Hai'an and Xinfeng, had a similar composition.

Zhang Zhongying and his colleagues say that the pollen present in the yellow rain reflected the types of plants flowering at the time. They say that bees harvest pollen from *Ulmus*, Gramineae and Compositae

in the autumn. Some yellow rain spots collected from the surface of ponds contained pollen from the water weed *Nymphoides* and a plant seen on river banks, *Chlamydomonas*. Bees require a good deal of water when they are at work. The scientists say that it would be difficult to explain the presence of the pollen of these last two species in yellow rain specimens if it were not related to the activities of bees.

Comparing pollen grains in excrement removed from the bees with that present in yellow rain showed that both had a similar percentage of damaged grains. Of 190 grains removed from the gut, some 12.1 per cent were damaged whereas 12.6 per cent of the 213 grains taken from yellow rain spots were damaged. Some 99 per cent of the pollen on the body surface of the bees was undamaged. Bees lack the intestinal enzymes capable of digesting pollen grains and extract the necessary protein, starch and fat from cracks in the wall of the grain.

Examination of the yellow rain spots is said also to have revealed the presence of other substances. The scientists say that some of these were filamentous. Algae and bacteria were also present. Finally, the scientists note that there were a large number of bees in the areas where yellow rain occurred.

It would appear from the contents of the Chinese paper that the phenomenon described in Northern Jiangsu is remarkably similar to that which occurs in

Laos and Kampuchea. Yellow rain, it seems, does exist and seems to be a natural phenomenon.

Meselson put forward this theory when he spoke at the annual meeting of the American Association for the Advancement of Science this summer. Following his address, the State Department countered by dismissing Meselson's theory as "the great bee caper", claiming that government scientists had rejected the suggestion that yellow rain was a "natural phenomenon".

None of this has worried Meselson unduly. He is all the more convinced that yellow rain is a natural phenomenon following the discovery of the Chinese paper. He believes that there could still be a problem of fungal toxin poisoning among the population of South-East Asia, but that this is probably due to the consumption of mouldy food.

Analysis of the mycotoxins in tissue samples removed during an autopsy carried out in Kampuchea on a Khmer soldier — allegedly exposed to a yellow rain chemical warfare agent five weeks previously — revealed higher concentrations of the toxin T-2 in the stomach and intestine than in other organs. Given the fact that the toxins have a relatively short half-life in animals, Meselson and three colleagues, in a letter in last week's *Science* (28 October, p.366) argue that the autopsy data would suggest that the soldier had eaten mouldy food a day or two before he died. The State Department has yet to react to this latest evidence and to Meselson's interpretation of events. **Alastair Hay**

Polish science

Travel still a problem

THE Polish Government's period of amnesty for Solidarity activists who had continued their activities during the period of martial law expired last Monday. According to Polish Government spokesmen, life in Poland is now virtually back to normal. For scholars employed by the Polish Academy of Sciences who wish to travel abroad, however, one innovation of the martial law period is still much in evidence — the special form which the intending traveller is obliged to sign.

The early clauses of this document are innocuous: name and academic qualifications, place of employment and so on. Point 7, however, demands an extremely detailed account of "purpose of travel and scientific aims", including, for example, the exact title of the paper to be read at a conference or the exact topics to be discussed during a visit to a laboratory.

By point 8, unreality creeps in. The applicant's boss has to authorize him or her to deliver the invited paper and conduct conversations on the approved topic. In point 9, the traveller's duties are further spelled out; the traveller is obliged, if something arises not covered by point 8, to

get in touch with the academy or the nearest Polish embassy or consulate before making any decision. The traveller, moreover, is bound to present a viewpoint in accordance with the *raison d'état* of the Polish People's Republic and to report in person or (if this is impossible) by telephone to the Polish embassy or consulate of any country visited. Finally, the document notes somewhat sinisterly that "the duties of the traveller after returning from abroad are governed by special regulations".

The traveller submitting this document deals as following with the ethical contradiction in his action: "You may ask how, by sending this letter, did I abide by the duties imposed on me by the document I signed. Well, I kept my word exactly to the extent that the Polish Government kept its word — signed in August 1980 — in December 1981. He who breaks his promises has no right to expect others to keep theirs. And I believe that informing my fellow scientists abroad that the persecution of free thought continues in Poland is in the best interests of Poland — even if not in the interests of the "Polish People's Republic". **Vera Rich**

UK nuclear waste

Land burial plans raise hackles

NOBODY, it seems, wants to live near a dump, least of all a dump taking radioactive waste. So the British Government's announcement last week of two sites in England to be investigated in detail as possible radioactive waste repositories has faced the Nuclear Industries Radioactive Waste Executive (NIREX) with an uphill battle to persuade local residents that they will not soon be glowing in the dark. First reactions from local councillors in both places have been hostile.

NIREX selected the two sites after desk studies of their geology and hydrology, the main requirements being stable strata and minimal groundwater flow. Convenient transport has been a further criterion. One site is at Elstow, near Bedford in central England, and would consist of a series of shallow trenches in a thick bed of clay. It would take low-level waste such as contaminated clothing and tools, as well as intermediate waste with a half-life of up to 30 years. Dumping of low-level waste in the Atlantic has now been abandoned because



ICI's mine at Billingham

of industrial action by transport unions and, more significantly, the existing shallow repository at Drigg near the Sellafield (Windscale) separation plant in Cumbria is expected to be full before the end of the century.

If NIREX gets its way, the Elstow dump will also take some intermediate-level waste such as neutron-activated metal components from reactor cores. This would be encased in blocks of concrete or other material and stored in deeper concrete-lined trenches. A dump at Elstow could take about 10,000 cubic metres of waste a year for at least 30 years. The site would eventually cover several hundred acres and would be covered with a concrete "intruder shield" before being landscaped. The objective is to ensure the integrity of the structure for several hundred years so as to guard against accidental intrusion even if records are lost at some stage.

The second more controversial proposal is for deep disposal of intermediate level waste, such as fuel cladding contaminated with long-lived alpha-emitters, now stored in concrete silos at Sellafield. The plan is to use a disused anhydrite mine under a

chemical factory at Billingham, Cleveland, in north-west England. The mine workings are at an average depth of 750 feet, in stable strata, and the anhydrite is exceptionally strong and dry.

It is expected that 4,000 cubic metres of long-lived waste will have been produced by the year 2000, including that accumulating on power station sites, but as the mine has a total volume of 11 million cubic metres, space will not be a problem. All waste destined for the deep repository would be encased in a special concrete and grouted into the mine chambers. The deep repository is intended to minimize the chance of accidental intrusion for the indefinite future.

Both sites will now be investigated in detail by NIREX. Limited planning inquiries will probably be necessary before on-site investigations can start. If the suitability of the sites is confirmed, NIREX will then have to run the gauntlet of more planning inquiries. Total cost is put in the region of £100 million.

The Billingham site is owned by Imperial Chemical Industries Ltd (ICI), which last week was ostentatiously unenthusiastic about the plan. The company says it would need to be fully convinced on safety and need before granting access, but that it will not obstruct the planned investigations. ICI is already sensitive to local feeling about the concentration of its own plants in the area. Local residents, knowing the site was under consideration, are already organizing protest groups. At Elstow, in contrast, local councillors were taken by surprise last week and complained that they had not been consulted. The Elstow site is owned by the Central Electricity Generating Board, a partner in NIREX.

Conveniently, generic safety objectives for land disposal sites were published last week by the National Radiological Protection Board. In the absence of internationally agreed guidelines for safety after repositories have been filled and sealed, the board has derived its own. The essential difficulty is that the exposure of people to radioactivity from a sealed repository would follow only from events with low probability but large consequences. Excluding such imponderables as meteorite impacts, the board follows the general principle that no future generation should be exposed to a risk greater than that now accepted. In order to allow for exposure routes and health effects not yet recognized, a further safety margin of a factor of 10 is proposed in a consultative document produced by the Department of the Environment.

If all the safety requirements can be met, NIREX hopes that the Elstow repository will be operational by the end of the decade, but declines to speculate about Billingham.

Tim Beardsley

Clinch River

Requiem for fast breeder

Washington

AFTER 12 years of bitter controversy, the United States appears at last to have decided against completing the Clinch River liquid metal fast breeder reactor. Despite strong support for the project from the Reagan Administration and Senate majority leader Howard Baker, the Senate voted last week by 56 votes to 40 against a last-ditch plan to continue with Clinch River, which officially ran out of funds at the end of September.

Senator Baker, who represents the state of Tennessee in which the demonstration reactor would have been sited, said after the vote that the Senate had spoken and that there appeared to be little hope of salvaging the project. A spokesman for the Department of Energy said the department was now planning an orderly termination at the site. Some \$1,700 million has already been spent on Clinch River and the estimated final cost had been put at more than \$4,000 million.

In a heated debate, Senator James McClure (Republican, Idaho), claimed that completion of Clinch River was essential if the United States was to achieve energy independence. He read a letter from President Reagan saying that it would be ironic, on the tenth anniversary of the 1973 oil embargo, if the United States failed to complete the project "at a cost equivalent to eight days of imported oil".

Most senators, however, appear to have been more influenced by the spiralling costs of the project and the reluctance of the industry to shoulder a larger share of the financial risks. A new financing plan, developed by the Department of Energy and the 753 utilities in the Breeder Reactor Corporation, was denounced by Arkansas Senator Dale Bumpers as a "sham" that insulted the intelligence of Congress.

Under that plan, the federal government would have contributed another \$1,500 million towards Clinch River while the private sector raised \$1,000 million in bonds. A study by the Congressional Budget Office concluded that the plan embodied "virtually no risks" for the private investors, since the federal government would be required to cover the full cost of the equity investment, as well as principal and interest payments on the debt, through tax benefits, project revenues and, if necessary, outright subventions. **Peter David**

Correction

The Mathematical Theory of Black Holes by Subrahmanyan Chandrasekhar was published this year by Oxford University Press, not by Cambridge University Press in 1982 as stated in *Nature* 27 October, p.760.

AIDS and confidentiality

SIR — The news item "Confidential matters" by Stephen Budiansky dealing with the Centers for Disease Control (CDC) (*Nature* 11 August, p.478) is an unfortunate distortion of the facts.

He erroneously states, "Incidents such as CDC's delivery of a list of names of AIDS patients to the New York Blood Center — perhaps by accident — have hardly allayed fears". CDC did not provide names to the "New York Blood Center" in general; the names were delivered by hand to a specific physician, in order to determine a crucial question: is hepatitis B vaccine safe? It was critical to determine whether hepatitis B vaccine recipients were at risk; therefore, under conditions of the Privacy Act, the names were provided under very strict procedures to be compared with the known recipients of hepatitis B vaccine in an earlier vaccine trial conducted among homosexual men in New York City. This was not done by accident, but intentionally and professionally in an attempt to resolve a critical question. This quick check allayed early fears that hepatitis B vaccine might be causing AIDS. I emphasize that it was done following the provisions of the Privacy Act and in a manner respectful of the many physicians, health officials and individuals who had placed confidence in CDC.

It is not correct to say we refused to supply information to a congressman. We only refused to supply him with the names of AIDS cases and personal identifiers in our records. On 10 May, a congressional aide requested full access to our records including the names of AIDS cases. We offered full access to the records if personal identifiers were first removed. From that time until 26 July, an exchange of letters attempted to resolve those differences until agreement was reached that names and personal identifiers would be removed before the files were seen by congressional investigators. Our files have subsequently been opened to the investigators.

All of this has been an effort in good faith to avoid releasing the names of AIDS cases. I know of no breach in that attempt. A rudimentary investigation could have prevented Mr Budiansky and *Nature* from publishing these inaccuracies.

WILLIAM H. FOEGE

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• Stephen Budiansky replies — Congressional investigators tell a different story. The aide who attempted to examine CDC's files in May says that CDC refused access to any documents having to do with AIDS, including policy, programme and budget documents unlikely to contain confidential patient information. "Their contention was that there were patient

names everywhere", the aide says; in fact, now that Congress has been granted access, only 31 documents out of scores examined by an investigator from Congress's General Accounting Office have turned out to contain patient information that CDC insisted on deleting. Many of these deletions involved not even patient names, but rather ages or geographical locations of patients. And contrary to Dr Foege's implication, congressional investigators say that at no time did they ask to see "names of AIDS cases". As for the CDC's supplying names of AIDS patients to the New York Blood Center, the point is simply that news of the incident, rightly or wrongly, intensified distrust of CDC within the homosexual community. □

Indian expatriates

SIR — The proposed creation in India of a "technology city" (*Nature* 29 September, p.350) for expatriate scientists returning to India to enable them to work in the sort of conditions they have become used to will not help in revitalizing Indian science. Science has taken gigantic strides during the past few decades mainly because of research in Europe and the United States. Unfortunately, India has not evolved a scientific culture and scientists in India are not terribly science-minded. No rationale can justify the hostile attitude of Indian scientists within India towards their countrymen working abroad. The latter have established their reputation in a highly competitive atmosphere through sheer dint of meritorious scientific research, but there is no competition among the scientists working in India.

The creation of a technology city (as I understand it) will mean establishing a scientific community totally divorced from the mainstream of Indian science. It is repeating an earlier mistake when several Institutes of Technology (IIT) were created divorced from the academic milieu of the country — the universities. Major resources were directed towards establishing these institutes, as a consequence of which Indian universities were starved of support. The new institutions could not satisfy the needs of scholarship and excellence in scientific research, while the universities, with high traditions of scholarship and learning, have been reduced to academic nothingness because of faulty educational planning.

The fact remains that when the British left India in 1947 they left behind a number of universities endowed with high reputations in scholarship and research. The universities of Calcutta, Madras, Bombay and Allahabad come to mind. It may not be out of place to note that Professor Erwin Schrodinger accepted the chair of physics at Allahabad University, but was unable to take up the post because

of the Second World War. It is saddening to see the condition of academic life in these universities: academic excellence has not been sustained, and continuing decline has resulted in a demoralized academic community.

Science cannot grow and prosper in isolation. Science has advanced in the West because there has been a nucleus of people working hectically together. Such a nucleus does not exist in India. Those opposing the return of Indian scientists are afraid that they will have to re-evaluate their scientific research, which they are conscious has very little importance internationally, nor even any particular relevance for the problems of Indian society. The tragedy of Indian science is that the people who control scientific circles are scientifically most incompetent.

What is the way out? The universities or other existing research centres should be allocated a certain number of positions in addition to those that exist, to be filled by expatriates only. Such positions should have support from the government not only for the incumbents' salaries but also to create up to date research facilities. This would help create a nucleus of meritorious scientific work and might also help improve academic life. Those who are already at work would benefit from the knowledge and experience of the new incumbent, while up to date research facilities would be a bonus.

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Pauling institute

SIR — Your article on the Linus Pauling Institute (*Nature* 303, 103; 1983) contains inaccuracies which I would like to correct.

In June 1978, when I was president, director, research professor and a trustee of the Linus Pauling Institute of Science and Medicine, Linus Pauling abruptly took various actions against me and my research work. During the succeeding four years, many of my research results on nutrition and cancer were suppressed.

The claim, attributed to Pauling in the *Nature* article, that I was awarded \$575,000 in the out-of-court settlement only out of "concern about the likely costs and trouble of a protracted hearing" is misleading.

The settlement document, which bears Pauling's signature, specifies that "\$425,000 is deemed to be in settlement of the libel and slander claims", "\$100,000 is deemed to be for reimbursement of attorney's fees and costs" and "\$50,000 is deemed to be in settlement of the contract and conversion claims". The settlement document states that none of the parties admits liability.

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Survival and revival: Chilean universities under Pinochet

In a personal account, Simon Litvak assesses the position of the scientific community in Chile after a decade of military involvement.*

I WAS working in my laboratory in the Faculty of Medicine at the University of Chile in Santiago, a few days after the 1973 putsch, when armed soldiers burst in searching for "documents, arms and political activists". We went through the humiliating experience of walking hands-up to the faculty soccer field where for a couple of hours we were surrounded by soldiers pointing at us with their automatic machine guns, while our laboratories, offices and scientific files were searched.

Those were very difficult years for the great majority of Chileans and the humiliation I experienced was very little compared with the suffering of victims of the repression which characterized the period between 1973 and 1977 in Chile. I remember, for instance, the case of a world known Chilean microbiologist who was arrested shortly after the putsch because an anonymous informant accused him of organizing a programme of bacteriological war on behalf of the Allende Government against the opposition forces. He was released only after intense pressure from high officials of the US Government. Other people working in the universities who did not have such support were less lucky. Many others left the country through the normal channels or after seeking refuge in Santiago's embassies. It is difficult to quantify such migration, but an approximate figure of 15 per cent has been given in the case of biological sciences. Significantly, many of the scientists who left the country were group leaders, able to find good positions abroad, leaving behind them leaderless laboratories.

Improvement

Repression has clearly decreased in recent years and since 1977 censorship of the media has been less harsh. With the arrival of the present Minister of Education some months ago the panel of military advisers to the universities was disbanded. Recently the minister declared publicly that no paramilitary guards should be seen in the universities. Another positive factor is the way that the scientific community has been able to block some of the most destructive measures from the government. The concerted effort of many leading Chilean



Pinochet (centre) surrounded by his military top brass

scientists led to the removal, some years ago, of a university president notable only for his blind admiration of the Spanish dictator Franco. More recently a well known biologist who was ousted from a university because of his critical opinions published in a weekly magazine, was reinstated in his job, after a strong reaction from Chilean scientists supported by many foreign colleagues. A further sign of improvement is the fact that the new university legislation committees are now working with impressive freedom towards a new "University Rule".

But the military presence has not completely disappeared from Chilean universities, as most of the people working there would prefer. Presidents of Chilean universities are named by Pinochet himself and selected from high ranking officials of the army, navy or air force. Once in position, however, a few of the military appointees begin to realize that the task of a university president goes beyond the mere application of the "law and order" doctrine. After some time in charge they become so involved with the university problems and defend the case of their employees so intensely that the government is compelled to change them.

A special case is the Catholic University in Santiago where the influence of the Church hierarchy is very strong. No change of president has occurred since the nomination of a naval officer in 1973. This stability is reflected in the better overall functioning of this university as compared with other centres. And the first civilian university president since 1973 was nominated recently for the Catholic university of Valparaíso. This is an extremely positive signal, provided that the civilians named are academics who can gather a

consensus from the diverse groups of people involved with the universities.

It is hard to tell whether the naming of the first civilian as a university president is a timely concession due to Pinochet's present defensive position, based on an increased dissatisfaction from all strata of the Chilean population, or a recognition of the special status of the Catholic universities. Alternatively, perhaps there is a genuine wish among government officials to return to a normal university life. In any case, there is a general recognition of the artificial nature of the military presence in the universities and of the need for change. Few would favour a return to the demagogic state of Chilean universities during the period before the putsch when the universities were in constant turmoil.

A public plea signed by hundreds of academics from all Chilean Universities was published recently asking for a rapid democratization and removal of military influences from the universities. This kind of declaration would have been unthinkable a year ago and reflects the fact that people are less scared now to criticize a more and more isolated government. The general impression that the universities are ready for constructive reform is reinforced by the fact that the atmosphere inside each faculty department is quite acceptable even though each department director is arbitrarily named by the faculty dean who is, in turn, named by the university president without consultation with members of the faculty. I have witnessed more open and constructive discussion of difficult problems in some Chilean university departments than in other countries respected for their democratic governments, but whose university departments are run in a much more dictatorial, truly

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"Pinochetian", style.

Research

In Chile most scientific research is performed in the universities. Biology was the first of the natural sciences to be developed in the nineteenth century with the arrival of foreign scientists at Chilean Universities; but only in the period between 1955 and 1970 did the work expand to a level comparable with research centres in more developed countries. The growth in research during that period was reflected in the statistics that show that, considering the country's population, Chile became the leading country in the number of publications in Latin America. One reason for this may be found in the return of many Chilean scientists who received their PhDs in the United States and were given leading responsibilities on their return. Also important was the fact that foreign agencies like the US National Institutes of Health, the National Science Foundation, the Ford Foundation, the Population Council, the Wellcome Foundation and others, supported Chilean projects and provided the money for heavy equipment, much of which is still in use. The increase in support from the Chilean Government for basic research in the 1960s was exemplified by the establishment of the Comisión Nacional de Investigación Científica y Tecnológica (CONICYT). This organization started by giving small research grants to promote the development of some research groups which were not funded by foreign agencies. Unfortunately in the years that followed, CONICYT lost most of its importance and very soon its budget had dwindled to a level that was sufficient only to pay the salaries of its employees. Since the early 1970s its role has been to administer the paltry funds supporting basic or applied research. It is to be hoped that the recent ousting of an army general named as president of CONICYT in 1973 will presage a renewed vitality of this essential organization.

The Allende era was characterized by a number of lyrical proclamations on the importance of developing basic and applied research in Chile, but very little was done to implement such proposals. The National Assembly of Chilean Scientists in 1972 (very similar to the French Assises Générales de la Recherche, gathered by the government of François Mitterrand in 1982 in Paris) was an interesting intellectual experience without concrete results. It can be argued, however, that it is unfair to judge a government on its record over such a short period and under very severe economic pressures.

Isolation

A critical point in the state of the research laboratories was attained in the first years after the putsch. To the important exodus of Chilean scientists mentioned above we have to add the international isolation of the military regime, the moral and eco-

nomic situation in the universities and the continued decline of CONICYT. The economic policies that prevailed in Chile until very recently, based on Milton Friedman's doctrine of free enterprise, had a disastrous effect on the universities. The government set out to reduce its support for the universities by encouraging their "self-financement". The severe cuts in their budgets diverted the attention of many researchers from academic and scientific matters to the problem of survival. These cuts were also reflected in the ousting of many people and by early retirements. The decrease in the size of the academic community resulted in an increased burden of teaching duties for those lucky enough to keep their jobs, and, of course, less time to pursue research. Another result of the Friedman philosophy was the sudden proliferation of private institutions given the rank of universities but with clearly lower standards than the established institutions.

The decline of CONICYT was in part counterbalanced by the creation of some university grants (US \$4,000 to 8,000 per year) that miraculously survived the Friedman era. More recently the National Research Fund (with similar grants) which supports basic and applied research in all scientific areas was created. Fortunately, at least for the time being, this fund is in the hands of respected scientists. It is obvious that with so little money available the main problem is the renewal of heavy equipment. The approval of a US \$65 million grant from the Interamerica Development Bank for the development of science in Chilean universities is expected in the very near future. This money should be used to improve buildings and to provide more heavy scientific equipment at various research centres. If this important opportunity is to be grasped, a serious and open debate in each faculty will be needed to ensure the money is properly distributed.

Doing research in countries like Chile is something very often beyond the comprehension of most of the researchers working in developed countries, who look upon the work of their colleagues from underdeveloped countries with a mixture of pity and open distrust. The amount of effort required to gather enough results to publish an article in a well known scientific journal is at least five times greater in a laboratory from an underdeveloped country than for others. A crucial factor that has helped to keep the flame of research burning in the stoic Chilean scientific community is the emergence in the mid-1970s of a United Nations Regional Programme for Post Graduate Training in Biological Sciences that covers most Latin American countries. Apart from providing some money for collaborative research grants, involving in each case two or three laboratories in different countries of Latin America, this programme has helped to support the visit

to Chile of many foreign scientists to assist in postgraduate courses and for national or international meetings. This, and the freedom now enjoyed by Chilean scientists to travel abroad, has allowed them to keep in constant contact with foreign colleagues.

The future

The quality of Chile's young scientists is still very high and many of those going abroad for their postdoctoral training are often attracted to and easily integrated in laboratories abroad. This dangerous "brain drain" has been reversed of late with the return of some senior scientists who, if given the chance, may help to revitalize research in the biological sciences. In discussions with people from the main groups involved in biological research in Chile I have noticed that the trials of these difficult years have yielded a degree of maturity in their projects. There are grounds for optimism. After a first phase which has seen the emergence of twenty or thirty laboratories involved in basic research in the biological sciences, publishing their results in the most cited international scientific journals, these groups, composed of a rich mixture of senior and young researchers, should develop an increasing interest in the study of applied projects. The worldwide explosion in biotechnology is now reaching some underdeveloped countries whose economies could most benefit from sensible use of biotechnology. This is reflected in several lines of research in Chilean laboratories, some already giving promising results. This biotechnological 'know how' will be of great importance in the production of Chilean raw materials, like minerals, cellulose and sea products, as well as in the study of diseases prevalent in this country. Crucial to the development of these projects will be the outcome of negotiations now under way to arrange for grants from foreign countries.

Viewed from abroad the political situation in Chile seems to be evolving very rapidly towards a return to democracy. However, inside the country the situation seems different. There is no doubt that the position of the current government is becoming weaker and weaker, mainly because of the enormous economic difficulties of the country, but no change can be expected without at least the tacit approval of the armed forces. Amongst university students there are signs of some unrest, with demonstrations staged on the 11th of each month to mark the anniversary of the overthrow of Allende. The survival of this healthy reflex in a generation raised since the arrival of the military regime is a very positive sign. We can be optimistic about the future of Chile's universities, provided that the nation's youth can pragmatically channel their longing for democracy into constructive areas, dismissing over-ambitious utopian dreams and the use of violence. □

Snowflakes are far from simple

Enough is known of the problem of how crystals grow to know that it will not be solved easily. But the outlines of an understanding are now apparent.

IF science cannot explain the patterns in which snowflakes grow, why should its practitioners be trusted on more complicated matters, the safety of nuclear power stations, or the pill? That is a particular version of a common more general gibe whose underlying error is sheer ignorance. If the problem of the growth of crystals were anything but complicated, why would people such as the late F.C. Franck (who died last year) have spent a patient lifetime on the explanation of a few facets of the host of phenomena he described? Moreover, the reasons the problem of the ice crystal is complicated are interesting and important (as metallurgists worrying about the limitations of zone refining will confirm). And there is a little progress to report: the accompanying diagram, showing successive stages in the computer simulation of the growth of a dendritic crystal (in two dimensions) demonstrates both that the essence of the problem may be understood — and that there is still a long way to go.

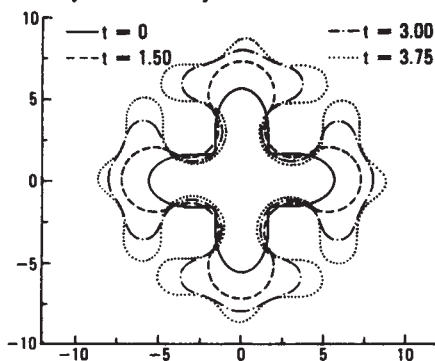
The problem of crystal growth is complicated for a very simple reason: it is a problem with nearly as many degrees of freedom as there are atoms or molecules on the growing surface. Moreover, the rate at which a crystal grows is determined not simply by the intrinsic properties of the material and of the phase (melt, solution or vapour) from which it is being formed but by an outwardly extraneous consideration — the rate at which the latent heat of solidification is removed from the surface.

Qualitatively and crudely, an ice crystal growing from a saturated vapour will grow most quickly at those points on its surface where the latent heat escapes most quickly, which will be the places where the radius of curvature is the smallest. But for a growing snowflake, the radius of curvature is least, or the curvature is greatest, precisely where rapid growth has already taken place, at dendritic tips. So positive feedback or the Matthew principle ("To him that hath . . .") applies, and dendritic growth as in a snowflake is a manifestation of instability. All this is elementary textbook stuff.

The formal analysis of the problem is inevitably more complicated (but J.S. Langer has given a remarkably clear account of it in *Rev. mod. Phys.* **52**, 1–28; 1980). To calculate the shape in which a snowflake grows from a pure vapour is to calculate the time-dependent shape of a surface whose motion is determined by heat diffusion at all points, both inside the

surface and in the region outside it, at least up the boundaries of the heat sink at which the latent heat is absorbed and whose temperature must be less than the melting temperature.

On the face of things, this part of the problem merely requires that the temperature everywhere should satisfy the equations of thermal diffusion and that something should be said about the temperature at the interface. If that temperature were the melting temperature, there would be little difficulty. The snag is that the temperature will not be constant but, instead, depressed from the melting temperature by an amount that is a function of the curvature and of the surface tension (or of the free energy of material in the surface). This phenomenon is the again familiar textbook explanation of why, in clouds, water vapour can be cooled below 0°C without condensing into ice crystals — vapour is stable with respect to very small ice crystals.



Mathematically, the dependence of the interfacial temperature on the local curvature is an immense complication. Physically, these two effects of curvature are antagonistic at a growing dendritic tip. Curvature certainly increases the ease with which latent heat fans out from the dendritic surface but, by reducing the apparent melting temperature, it also flattens the temperature gradient and thus reduces the specific heat flux. But when the curvature is high, the second effect swamps the first, so that very fine dendritic tips, like flat surfaces, will lose heat (and thus grow) only slowly. And at some intermediate curvature, the rate of heat loss, and thus the speed at which the tip will grow, will be a maximum.

That, too, would be good textbook material if there were some reason to believe that reality is as simple. But dendritic tips do not grow indefinitely nor (as

J.S. Langer points out) as quickly as the calculations suggest. Instead, as the shape of every snowflake shows, dendrites grow side branches behind the growing tip. The diagram is from a paper by R.C. Brower, D.A. Kessler, Joel Koplik and Herbert Levine (*Phys. Rev. Lett.* **51**, 1111–1114; 1983) whose chief purpose is to demonstrate the emergence of side-branched dendrites in a computer solution of the equations governing the process of solidification. Simple shapes (like the bulbous cross at the centre) are unstable relative to more complicated shapes, while the problem is non-linear. Rapid growth, as in the formation of a dendritic spur, entails a kind of controlled instability. Oddly, nobody has yet found a way of incorporating the effects of crystal anisotropy (which must be why snowflakes have hexagonal symmetry).

So why not start at the other end of the problem, where growth is slow or even non-existent, by building in the lattice properties of the solid? It should then be possible to tell the shapes that crystals take simply by minimizing the surface energy of some specified bulk of material. The snag here is that of calculating the surface tension or, which comes to the same thing, the exceptional free energy of atoms or molecules in or near the surface. There has been some success with unrealistically simple models such as the Ising lattice — but now D.B. Abraham (*Phys. Rev. Lett.* **51**, 1279–1281; 1983) claims to have found a neat way of calculating surface energy by means of statistical argument based on the topological similarity between stepped terraces on the surface of a growing crystal and the six-vertex lattice problem that crops up both in the calculation of thermodynamic properties on a lattice and in the renormalization of the fields of quantum chromodynamics.

Where all this will lead is anybody's guess. Both Abraham's calculation and that of Brower *et al.* are recipes for further work to do. But the importance and the ubiquity of the problem is not in doubt: a group from the *Université de Provence* (Searby, G., *et al. Phys. Rev. Lett.* **51**, 1450–1453; 1983) has just described some neat observations of the stability of a plane flame front in a moving stream of combustible gas which demonstrate that to be analogous to the chemical stability of a zone-refining procedure except that hydrodynamic forces play the part of surface tension.

John Maddox

Neurosciences

How molecular is neurobiology?

from Colin Barnstable, Thomas Jessell, Joshua Sanes, Charles Stevens and Miranda Robertson

COLD Spring Harbor Symposia have established a tradition of being in at the birth of new biological sciences; and this year's main symposium* acknowledged the arrival of molecular neurobiology, born of the union of recombinant DNA and the conventional neurosciences, with help from monoclonal antibodies. At this stage however it could be argued that the resultant progeny is more in the nature of a mosaic than a true hybrid. While neurobiologists at the meeting were still raising important questions that have no molecular answers, many molecular biologists were finding themselves in the curious position of having arrived at answers for which they now have to discover the appropriate question. In the highly selective account of the meeting that follows, both of these dilemmas will be illustrated, along with the one instance in which neuro- and molecular biology have achieved genuine fusion: the study of the acetylcholine receptor.

Neuropeptides and other mysteries

A particularly vivid illustration of a molecular answer in search of a neurobiological question is to be found in the work of J.G. Sutcliffe, R.J. Milner and their associates at the Salk Institute. Their approach has been to clone cDNAs transcribed from total brain polyadenylated RNA and then establish which of the mRNAs is synthesized exclusively in the brain¹. This strategy has produced more than 50 brain-specific cDNAs encoding molecules of unknown function.

Some of the more abundant products may reasonably be expected to be peptide neurotransmitters, and on the basis of this expectation Milner has been conducting electrophysiological experiments with synthetic peptides corresponding to selected sequences within one of the larger cDNA clones. The sequences chosen were those that fell between nucleotides encoding pairs of basic peptides that might plausibly represent cleavage sites for generating separate peptides from a polypeptide precursor. Antisera raised against three such peptides turned out to stain nerve fibres in the cerebellum, cerebral cortex and hippocampus, and one of them proved on electrophysiological testing to excite cerebral cortical neurones and inhibit hippocampal ones. The next question is that of the physiological function of the peptide; and it will require some ingenuity to generate an unambiguous answer.

This approach thus raises in a particularly acute form the general problem — explored recently in *Nature* by Peter Newmark² — of how to find out the physiological significance of new peptides revealed by cloning the DNA encoding the known ones. A major handicap is the com-

plexity of the mammalian central nervous system: the spectacular success of R. Axel, R.H. Scheller and their collaborators at Columbia University, using the same general approach, is due to their decision to work on the peptides of the sea snail *Aplysia*, whose nervous system is not only relatively simple but exceptionally well charted. This has recently enabled them to make at least an intelligent guess about the evolutionary and physiological significance of a family of peptides related to the known egg-laying hormone³; and now Scheller (Stanford University) has demonstrated that it is possible to use the same cloning techniques to isolate peptide genes in secretory neurones without any prior knowledge of the products.

The *Aplysia* abdominal ganglion contains several identified neurones that are considered, on the basis of their ultrastructural morphology, to have a secretory function. One of these neurones, R14, is known to synthesize large quantities of a 12,000-molecular weight (MW) protein which is subsequently processed into an acidic 3,800-MW peptide and a second basic 5,000-MW peptide⁴. Scheller has now isolated mRNA from these neurones and generated labelled cDNA probes with which to screen an *Aplysia* genomic library. This has revealed several clones hybridizing specifically to R14. The R14-specific genomic clone produces a primary translation product containing acidic and basic proteins similar to those demonstrated by biosynthetic labelling. The same precursor protein also contains another 12-amino acid peptide that is flanked by pairs of basic amino acids characteristic of sites of proteolytic processing. The sequence of this peptide corresponds precisely with the partial sequence data available on a peptide isolated from *Aplysia* abdominal ganglia by Mayeri and his colleagues at the University of California in San Francisco, who have shown that it has pronounced excitatory actions on a network of abdominal ganglion neurones and their target cells. Related transcripts appear in other *Aplysia* cells, so once again this gene seems to be part of a larger family.

The evolution of multiple germ-line genes represents one mechanism for generating diverse but related peptide hormones. An alternative is the differential splicing of RNA transcripts, recently shown by R. Evans (Salk Institute), M.G. Rosenfield (UCSD) and their colleagues in the case of the calcitonin gene. Their starting-point was the observation that tumours derived

from medullary thyroid carcinoma cells normally secrete large amounts of calcitonin but occasionally switch to the production of a larger transcript that still contains calcitonin gene sequences but does not produce the calcitonin peptide⁵. Evans and Rosenfield have been able to demonstrate by means of cDNA probes that the two mature RNA species are generated by differential processing of the same primary transcript⁶. The second mRNA encodes a 128-amino-acid, 16,000-MW protein that has N-terminal homology with the calcitonin precursor but substitutes the entire calcitonin-containing C-terminal region with a sequence containing a new 37-amino acid peptide named calcitonin-gene-related peptide (CGRP)⁶. CGRP represents another peptide looking for a function. The only clue so far comes from antisera raised against synthetic CRGP, which have revealed that CGRP is a major new neuropeptide present in both sensory and motor neurones and several ascending central pathways. But CGRP immunoreactivity is also detectable in the adrenal medulla and endocrine pancreas, suggesting that it may function as a circulating hormone as well⁷.

The differential production of peptides from polypeptide precursors presumably depends on the regulation of the enzymes that cleave them. There are at least two major classes of such enzymes: the arginyl proteases, which are important in the processing of opioid peptide precursors^{8,9} and pancreatic prohormones¹⁰; and the kallikreins, which seem to be involved in the activation of several peptide growth factors and vasoactive peptides. The genes encoding the kallikreins have recently been isolated by J. Shine (Australian National University), and turn out to consist of a large family of 25–30 members with an overall amino-acid homology of about 75 per cent¹¹. The greatest sequence divergence is found at sites that are thought to contribute to the catalytic activity of the enzymes, and may thus be the basis for differences in their substrate specificity. Very little is known so far about the individual enzymes, and the use of recombinant DNA should be invaluable in establishing how the production of different peptides from the same precursor is differentially regulated in various tissues.

Neural recognition and embryogenesis

If molecular biology has not yet made major inroads on the understanding of neural physiology, what promise does it show on the question of neural development? Much of the hope of progress has been pinned on the use of monoclonal antibodies to identify molecules that mediate cellular interactions. Once these molecules have been identified, it has been argued, it should be possible to isolate the genes that encode them and to learn how they may contribute to specific neural connectivity. Several speakers described the first step in this approach: the immunological identification of markers for specific cells types (or

*The Cold Spring Harbor Symposium on Molecular Neurobiology was held on June 1–8, 1983.

subsets) in the leech nervous system (B. Zipser and R. McKay, Cold Spring Harbor Laboratories)¹², in the cat visual cortex (S. Hockfield, Cold Spring Harbor Laboratories) and in the retina of rat (C. Barnstable, Rockefeller University) and *Drosophila* (S. Benzer, Cal Tech).

Before the promise of the approach can be fulfilled, however, there are two problems to be overcome. First, some way has to be found of discovering how important the surface markers are either in defining or in determining the different phenotypes of neurones; and second, few have yet been properly characterized biochemically.

Furthermore, in the case of developmentally regulated surface molecules an additional complication arises. It is widely believed that many cell-cell recognition mechanisms that guide development are mediated by adhesive interactions between cells. The best candidate for a role in such interactions is the so-called neural cell adhesion molecule (N-CAM), a surface glycoprotein that causes the aggregation of nerve cells and fasciculation of their processes *in vitro*^{13,14}. Related antigens have been independently identified by several groups and variously named D2 (ref. 15), BSP2 (ref. 16), L2 (M. Schachner, Heidelberg University) and 22A1A6 antigen¹⁷. This molecule exists in at least two forms, embryonic and adult, which have different adhesive properties. The complication lies in the fact that the difference between embryonic and adult forms is due to a difference in glycosylation. This means that developmental regulation is acting, not on the gene specifying the surface protein but on the glycosylation mechanism. Thus it may be the glycosylating enzyme that is developmentally regulated; or — since the glycosylation of proteins depends on their entry into specific compartments of the endoplasmic reticulum — regulation may entail the redirection of the protein through this cytoplasmic labyrinth. Little is known of the glycosylating enzymes and even less of cytoplasmic traffic control; so the genetic basis of these developmental modulations may prove exceedingly difficult to establish.

At the same time, it remains to be demonstrated that the N-CAMs play a part *in vivo*. Some headway in this direction has been made by U. Rutishauser (Case Western Reserve University) who, with F. Bonhoeffer, has shown that injection of N-CAM into the embryonic chick eye disorders the axons growing out from the retinal ganglion cells, though it does not prevent their outgrowth. It remains to be discovered whether N-CAM is an all-purpose glue that permits more subtle interactions, or whether it is itself involved in the choices that axons make among pathways and targets as they grow.

Similar unresolved questions arise in connection with developmentally interesting components of the cell surface that are not integral to the membrane, but are associated with the extracellular matrix. At

the neuromuscular junction, for example, the basal lamina that extends through the synaptic cleft can organize pre- and post-synaptic differentiation when nerve and muscle regenerate after damage (U. McMahan, Stanford University; Sanes, Washington University, St Louis). Furthermore, materials secreted by cultured cells that mediate adhesion or promote neurite outgrowth *in vitro* contain molecules common to many extracellular matrices: a heparan sulphate proteoglycan (L. Reichardt, UCS, collagen and fibronectin (D. Schubert, Salk Institute). McMahan has obtained an antiserum to a matrix-derived protein from *Torpedo* electric organ that blocks matrix-induced clustering of acetylcholine receptors on cultured myotubes; and P. Patterson and W.D. Matthew (Harvard University) have used immunization *in vitro* to produce a monoclonal antibody that blocks neurite outgrowth induced in sympathetic cells by a heparan sulphate proteoglycan. Once again, it remains to be shown that they appear at the appropriate time in development *in vivo*, and to establish that they influence developmental processes.

As with neuropeptides, these questions may prove easier to resolve in invertebrates — nematodes, insects and leeches — whose simplicity facilitates descriptions of extraordinary detail and completeness. For example, D. Bentley (University of California, Berkeley)¹⁸ and C. Goodman (Stanford University) can watch axons of identified neurones grow along defined pathways to their targets in the nearly transparent grasshopper embryo. The first neurones to differentiate commonly put out processes that act as guides to later-developing axons, and themselves use other neural cells as 'guideposts' determining the bending and branching of their paths through the embryo. From the pathways that axons follow and ignore, and from disturbances in pathfinding caus-

ed by ablation of putative guides, it is possible to begin assembling cellular and subcellular explanations of some aspects of development at a level that is still unthinkable in vertebrates.

Two recent developments offer the exciting prospect of using classical developmental genetics in combination with monoclonal antibodies to fulfil the potential everyone has seen in the use of these markers. Goodman has been able to use monoclonal antibodies to identify specific axons bundles early in development, while at the same time R. Wyman (Yale University) has isolated *Drosophila* mutants (named *bendless* and *passover*) in which the branching pattern of one or a few axons is altered — possibly because of failures in pathfinding during embryogenesis. Since *Drosophila* and grasshopper nervous systems are similar and develop similarly, the exciting prospect arises of exploiting the *Drosophila* mutants to establish whether the antibodies that react with grasshopper axons are in fact identifying developmentally regulated molecules that determine the paths taken by insect nerves — and ultimately thus to isolate the genes controlling these events.

Channels of communication

For the present, the peak of the actual achievement of molecular neurobiology remains the cloning of the genes encoding the four subunits of the acetylcholine receptor (AChR), the best understood of the many integral membrane proteins that form the ionic channels underlying the electrical activity of the nervous system¹⁹.

S. Heineman and J. Patrick (Salk Institute), E. Barnard (Imperial College, London), and J. P. Changeux (Institut Pasteur) have cloned and sequenced one of the four subunits (α , β , γ , δ) that comprise this protein, and S. Numa and his colleagues (Kyoto University) have quite recently published the complete sequence of all four. One important aim in sequencing channel proteins is to define relationships between the various channel types: gene families, such as that for globin, are generally expected. So far none has emerged: Numa's and N. Davidson's (Cal Tech) laboratories report evidence for only single copies of genes for two of the subunits. This result was somewhat unexpected because physiological and biochemical evidence indicates the presence of at least two forms of the AChR during development (one found before synapse formation and the other after) and the parallel with fetal and adult forms of globin, each with its own genes, was appealing. Not only is evidence for multiple forms of AChR genes lacking, but Hall presented results suggesting that, as with N-CAM, at least some of the developmentally related changes in the AChR arises from addition or modification of sugars attached to the α subunit. If the genes for the AChR are part of a family, then, it is not a close-knit one. Homologies between members of such a possible family must be

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relatively weak, at most on the order of similarities between the different subunits themselves (up to about 70 per cent DNA homology).

Meanwhile, the biochemistry of the sodium channel is advancing rapidly, and representatives from three laboratories (W.A. Catterall, University of Washington, Seattle; W.S. Agnew, Yale University; L.C. Fritz, Cal Tech) agree that the principal 260,000-MW peptide chain forming this channel can be purified and recognized by antibodies. Catterall finds, in addition to the large peptide, two smaller associated peptides, but Agnew detects only a single large chain. As the smaller peptides are found in mammalian sodium channels but not in eel, species differences may well be responsible for these divergent findings, a possibility supported by the observation that antibody cross-species reactivity is slight or absent (Fritz). With antibodies that recognize sodium channels available, and with amino acid sequence data soon to come, several laboratories are hoping to clone sodium channel cDNA, within the next year.

Sequence data are the first payoff from cloning channel genes; one of the later prizes will be the ability to probe function by using site-directed mutagenesis and chimaeric genes for channel DNA expressed in clonal cell lines. A glimpse of this goal was provided by studies using DNA coding for the bacterial aspartate receptor mediating chemotaxis (D.E. Koshland, University of California, Berkeley), and for bacterial rhodopsin (H.G. Khorana, MIT). Although modifying proteins selectively by altering their DNA has not yet unravelled the functional mysteries of these proteins, the method works.

Once the same approach can be used to investigate major neurotransmitter receptors other than the AChR (which has already been produced in functional form in the membrane of oocytes from messenger RNA)²⁰, neurophysiologists and molecular biologists will be able to collaborate on the kind of question to which the molecular approach can at last give unambiguous answers: for example, are there two, three or four subtypes of dopamine receptor or are some of them artefacts of the indirect techniques now needed to identify them?

Given a biochemical handle on one or two of the putative receptor families, molecular biologists may be able to do for neuropharmacology what they have already begun to do for immunology. □

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Biochemistry

Inositol trisphosphate: link or liability?

from Robin Hesketh

THE question of whether an increase in the metabolism of inositol lipids in mammalian cell membranes is responsible for increasing the level of cytosol calcium as a second messenger has plagued cell biology for years. Calcium is implicated as an intracellular second messenger in the action of a large range of agonists, including classical neurotransmitters, peptide hormones and lectins, and in all such cases the agonists also stimulate the hydrolysis and resynthesis of phosphatidylinositol (PI). Although other putative second messengers may be generated, for example, cyclic GMP or activated protein kinase C, the important point is that whenever the agonist increases the concentration of free calcium within the cell, PI metabolism is stimulated.

Michell¹ suggested that the interaction of agonist with receptor on the cell surface caused PI hydrolysis which in turn resulted in the opening of gated calcium channels in the plasma membrane, enabling calcium ions to move down the concentration gradient into the cell. Over the last two or three years, however, a number of cell systems have been reported that appear not to conform to this simple hypothesis. In some, stimulation of phosphoinositide metabolism appears to be totally dependent on extra-cellular calcium and PI breakdown can be induced by the calcium ionophore A23187 (see *News and Views*, 295, 281; 1982). In other cells the source of calcium appears to be intra-cellular (that is, from the mitochondria or endoplasmic reticulum); examples now include the stimulation of pancreatic secretion by acetylcholine, the system in which the Hokins first detected the PI response in 1953². It should be made clear, however, that the complexity of the PI cycle renders PI labelling data ambiguous and breakdown studies have been unable to refute or confirm Michell's model³.

Demonstrating that any theory is capable of rapid mutation to accommodate nonconformist data, Michell and his colleagues have recently suggested⁴ that it is the breakdown of the phosphorylated derivatives of PI, PI-4,5-phosphate and PI-4,5-bisphosphate, rather than PI hydrolysis, that increases the level of calcium. These derivatives comprise only about 10 per cent of the total inositol lipids and arise from the sequential actions of kinases that specifically phosphorylate hydroxyl groups on the 4- and 5-positions of the inositol moiety of phosphatidyl inositol. The hydrolysis of each of these lipids is brought about by phosphodiesterase action, releasing diacylglycerol

and inositol-1,4,5-trisphosphate (ITP) from PI-4,5-bisphosphate. There is evidence^{5,6}, albeit not clear-cut^{7,8}, that these reactions do not require a rise in the resting level of cellular calcium and could therefore constitute the signal for calcium release and subsequent second messenger activation. Using a variety of chromatographic and ionophoretic methods it is now possible to quantify agonist-induced changes in the levels of inositol phosphates⁹. When insect salivary glands are stimulated with 5-hydroxytryptamine the concentration of ITP rises dramatically within seconds and Berridge¹⁰ has proposed that this is responsible for elevating the intracellular free calcium concentration. Although the ITP will ultimately be utilised for resynthesis of PI, Berridge suggests that it should be doing something useful meanwhile.

The first evidence that ITP might be the key which releases intracellular calcium has now appeared in an intriguing paper by Streb *et al.* published in this issue of *Nature* (p.67). The plasma membranes of pancreatic acinar cells were made 'leaky' by washing in a nominally calcium-free buffer. The cells were then incubated in a high potassium medium with respiratory substrates and ATP. The leaky cells eventually reach a steady state with respect to calcium distribution at an external free calcium concentration of about 0.4 μ M. Addition of ITP then causes a transient rise in extracellular calcium. This increase is independent of the presence of inhibitors of mitochondrial respiration, suggesting that it represents the release of calcium from non-mitochondrial stores, followed by slow re-uptake to the original steady-state level. These 'leaky' cells still respond to carbamylcholine by releasing calcium in an effect which is completely inhibited by exogenously added ITP, indicating that the same calcium reservoir is the target of both agents. Other phosphoinositides were without effect and the specific activity of ITP appears to derive from the arrangement of the three phosphates on the inositol ring.

Although these findings do not prove that ITP is a second messenger for internal calcium mobilisation, the ITP concentration required for release (1–5 μ M) is of the order estimated to be present in stimulated parotid cells⁶ or basophils¹¹. It is tempting therefore to argue that one of the products of the calcium-independent hydrolysis of PI-4,5-bisphosphate, ITP, can change free calcium levels in cells. Diacylglycerol, which is also formed during the hydrolysis,

is known, at least in platelets, to activate protein kinase C¹². The synergistic effects of increase in free intracellular calcium and the calcium-independent activation of protein kinase C have been clearly demonstrated in the platelet^{13,14} and it is this kinase, of course, that is activated by and appears to be a receptor for tumour promoters such as tetradecanophorbol acetate (TPA)¹⁶. It is an attractive notion that synergism between calcium-dependent and calcium-independent events may be a general feature of cellular control.

The critical question still remains: does the breakdown of phospholipid *in vivo* precede and control the elevation of intracellular calcium or are the responses essentially independent? The extreme rapidity of the ITP increase in blow-fly salivary gland, which clearly precedes an increase in ⁴⁵Ca²⁺ flux across the cell, is consistent with the former, but in this tissue only PI-4,5-bisphosphate is hydrolysed by a PI phosphodiesterase and hydrolysis of the other phosphoinositides is contingent upon their previous conversion to the bisphosphate. In other cells, for example, 2H3 basophils¹¹, however, each of the phosphoinositides appear to act as substrates for one or more PI phosphodiesterases. In these cells a large increase in intracellular calcium, measured by the fluorescent indicator quin 2, is maximal within three minutes of stimulation, by which time there is only a small increase in the level of ITP. The levels of all of the inositol phosphates continue to rise thereafter, even though the free calcium concentration actually falls. In 2H3 cells therefore, the evidence that ITP regulates calcium looks much less convincing than in the salivary gland. The fact remains that although the calcium signal can be inhibited in 2H3 cells without affecting PI stimulation, the converse requirement, necessary to confirm independent

mechanisms, has not been demonstrated. Comparison of the kinetics of calcium and PI responses in different cell systems has not revealed any consistent patterns, perhaps simply reflecting phenotypic variation in the extent to which calcium-independent or calcium-dependent events predominate during stimulation. The final resolution of the relationship between PI and intracellular calcium levels will

probably need to wait for the production of specific inhibitors of the PI cycle.

If ITP does eventually turn out to be the 'missing link' between PI stimulation and calcium, as Streb and his colleagues suggest, the relief will be great, not only from those who have stuck to PI through thick and thin but also from those of us who simply want to know how calcium signals are generated. □

Ecology

Ecological diversity and stress

from Peter D. Moore

EVIDENCE for the notion that ecological systems are most diverse when they are subjected to a certain degree of stress is now being accumulated from a wide range of habitats. Stress can take the form of a physical limitation to overall productivity or of a degree of disturbance which prevents the assumption of dominance by certain aggressive or highly productive species. The principle has been stated in various ways¹⁻⁴ and has been illustrated in habitats as diverse as terrestrial grasslands and intertidal zones. In two recent studies, sites were artificially manipulated to introduce various forms of stress and the effect monitored. The results ably illustrate the complexity of competitive interactions in plant communities.

One of the studies was conducted in sub-alpine meadows on the high slopes of the Olympic Mountains in the western United States⁵. In these grasslands, overall productivity is related to such factors as soil nutrients, moisture and temperature. Diversity (measured by the Shannon-Wiener index *H'*) shows maximum values where there is moderate stress and thus where total productivity is sub-optimal.

Transplant experiments were used to reveal the role of competition in the reduction of diversity in the most mesic conditions. It was found that species transplanted from high-diversity sites to highly productive sites generally survived well if surrounding plots were cleared of competitors, but succumbed rapidly if the plots were uncleared. Seedlings were particularly sensitive to competition.

Certain species were then selectively removed by clipping and the reaction of the remaining plants monitored. The effects of removal of the dominant species were most marked in the more productive (less stressful) habitats. Occasionally the removal of one dominant, such as the sedge *Carex spectabilis*, simply permitted another plant to assume dominance, such as the grass *Festuca idahoensis*, often with a higher resultant productivity. Nutrient additions simply served to enhance the dominance of the most competitive species, where such a species was forthcoming, and hence reduce diversity further. These findings are in keeping with the ideas of Grime⁴ and of

Tilman⁶ who proposed that decreasing nutrient supply leads to increasing species richness until the point is reached where nutrients become limiting to the survival of coexisting species.

A comparable situation exists when the stress upon the plant community is due to grazing pressures. Paine⁷ proposed a concept of 'keystone species' in which the keystone is a predatory species which controls the population density of its prey and may thus prevent it from dominating a community through appropriating an undue share of resources. Hixon and Brostoff⁸ have now described a more complex situation in which a predatory damselfish which inhabits Hawaiian coral reefs controls the populations of herbivorous fish which in turn influence the algal components of the reef ecosystem. The relationships were examined by placing artificial settling surfaces in three types of site: within damselfish territories, outside territories and in fish-exclusion cages. After a year, the greatest algal diversity (*H'*) was found inside the damselfish territories and the lowest outside; caged samples were intermediate in diversity. Thus a high grazing intensity and an absence of grazing both resulted in lower algal diversities than intermediate grazing levels where damselfish controlled the population of grazing fish.

In effect, the grazing stress here is equivalent to the nutrient stress in the montane grasslands: increasing stress restricts the tendency for resources to be channelled into certain potentially dominant species and thus increases community diversity up to the point where tolerance limits of the more sensitive species are reached and extinctions commence. As far as diversity is concerned, a little stress can go a long way. □

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Geology

A new data source for *in situ* stress field orientations

from Mark D. Zoback

THE lack of reliable data that has hampered attempts to understand the nature and origin of stresses in the Earth's crust may soon be made good by a new method for studying the orientation of horizontal stresses. Easy to use wellbore logging instruments make it possible to determine the direction of stress-induced wellbore spalling which, for reasons explained below, takes place preferentially around the wellbore at the azimuth of the least horizontal stress. The method is now beginning to be widely used (see *Nature* 305, 619; 1983) and may well yield a wealth of data in a few short years.

Three main types of stress field indicators have been used in the past, but each type has distinct and well known problems. Earthquake focal plane mechanisms have been most commonly employed but, unfortunately, even well-constrained focal mechanisms for a given earthquake yield P- and T- axes which only approximate the principal stress directions. The determination of principal stress orientations can be improved by using a family of mechanisms from different faults in a given area, but available cases for such analyses are rare.

In situ stress measurements have also been widely used as stress-field indicators. Although shallow measurements are relatively inexpensive and easy to make, it now seems clear that reliable tectonic stress directions often must be taken from measurements made at depths of 50–100 m or more. Thus, strain relief stress measurements in mines (when the measurements can be made far enough away from the mine cavity) or hydraulic fracturing stress measurements in wells or boreholes are required.

Finally, geologic features such as dike and cinder-cone alignments, and fault-slip (slickenside) data on young scarps can also yield data on the orientation of the stress field. Well-exposed and sufficiently young (usually less than a few million years old) volcanic features cannot, however, be found in most regions of interest. Fault slip data have the same type of uncertainty as focal plane mechanisms, but again adequate data sets for a family of faults are rare.

It was noticed almost twenty years ago at the Nevada Test Site that wellbore elongation seemed to be controlled by the *in situ* stress field. The nature of this phenomena, however, is only now coming to light, largely through the work of two Canadian scientists; Ian Gough and Sebastian Bell (see *Nature* 305, 619; 1983). They showed that in several oil fields the well-

bore elongated in the direction of the least compressive stress as indicated by other stress field data in the region. These elongated sections of wellbores are often called breakouts in the petroleum industry. My colleagues and I at the US Geological Survey have recently substantiated the correlation suggested by Bell and Gough by showing that breakout and hydraulic fracturing data from the same well give the same stress field orientation at several different sites.

The devices that are now being used to study stress-induced wellbore elongation are four-arm oriented calipers and acoustic borehole televiwers. The caliper data are more exciting because information is already available for many wells and logs are commercially available in new wells. There are three problems with the breakout data yielded by the calipers, however.

First, the caliper only provides the minimum and maximum well diameter and no detailed information on the shape of the hole. Second, the caliper's low sensitivity means that many small breakouts may be

missed. Third, because of rotational torque on the caliper from the logging cable, a bias in the data can occur if the caliper arm does not stay centered in the breakout as the caliper is pulled up the hole.

The acoustic televiwer is a sophisticated ultrasonic scanning device which can be used to determine accurately the cross-sectional shape of wellbores. Although it does not have any of the disadvantages of the calipers, televiwer data are often difficult to obtain.

It appears that the origin of the breakouts is related to a compressive shear failure process because the breakouts form where the compressive circumferential stress concentration around the well is highest. As this point is located at right angles to the direction of maximum horizontal compression, the wellbore elongates in the direction of the least principal stress. Although the formation of breakouts has been addressed with limited theoretical and laboratory studies, a great deal more effort is still needed. With sufficient understanding, however, it may even be possible someday to estimate the magnitude of *in situ* stress from accurate measures of breakout size and shape. □

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Meteoritics

New isotopic evidence of early Solar System processes

from Mark H. Thieme

METEORITES possess the most pristine record of the early Solar System, since other planetary objects, like the Earth and Moon, have undergone secondary events that have erased the primordial record. Cosmochemists are particularly interested in meteoritic isotopic distributions, since they indicate the nuclear and chemical events associated with their own history and, presumably that of the Solar System at large.

In the latest issue of *Nature*, Lewis *et al.* have reported some striking new measurements for the isotopes of nitrogen in the carbonaceous chondrites Allende and Murchison. The reported measurements are of special significance, because of their apparent correlation with documented isotopic anomalies in neon, xenon and carbon and, hence, may be of utility in resolving their astrophysical origin. The peculiar reported isotopic signatures may, in fact, represent a mixture of a "normal" Solar System material and a component of material with an origin outside the Solar System.

Nitrogen possesses two stable isotopes, ^{15}N and ^{14}N , with natural abundances 0.36 and 99.64 per cent respectively. By convention, isotope geochemists report isotopic anomalies in the delta (δ) notation, where δ ^{15}N refers to the $^{15}\text{N}/^{14}\text{N}$ ratio in a sample with respect to the same ratio in a standard; for nitrogen this is air. The δ ^{15}N then is simply the proportional difference between a sample and air, expressed in parts per thousand, or per mil (‰). A positive delta is referred to as being heavy (^{15}N enriched) and negative as light, or ^{14}N enriched. On Earth, nitrogen isotopic compositions range from a δ ^{15}N of approximately -10‰ for natural gas to about +20‰ for nitrogen in mid-ocean ridge basalts. Lewis *et al.* report a nitrogen component in the Allende meteorite with a δ ^{15}N of -326‰, the largest ^{14}N enrichment ever observed. Even more impressive are the results from Murchison. Through chemical isolation of exceedingly rare components, they have shown the presence of several nitrogen components which range in δ ^{15}N from -272‰ to +153‰.

Before the appearance of this report, Thiemens and Clayton² had demonstrated that Allende possessed a light (-90‰) nitrogen component. Kung and Clayton³ had reported a heavy nitrogen ($+72\text{‰}$) component in Murchison, but there was no direct observation of the reported extraordinary species. Lewis *et al.* used an extensive and complex chemical procedure that, primarily through a sequence of acid dissolutions and oxidations, isolates a carbon-rich fraction contributing less than 0.2‰ of the original material. Further resolution is achieved by regulated vacuum stepwise heating of the residues that selectively releases volatile components as a function of their activation energy. The technique is extremely informative, since it isolates extraordinarily rare components which are normally masked by larger indigenous ones. In addition, the technique yields information on their chemical siting which is of importance for delineating the chemical, as well as nuclear, histories of these species.

This particular approach has been successful in resolving noble gas isotopic anomalies [see, for example, ref. 4]. Xenon and neon isotopic analyses in these acid resistant residues have revealed the presence of three contentious and presumably exotic components. The authors, as well as other meteoriticists, had previously suggested that these components may be: (1) a fission component, possibly derived from an extinct superheavy element, recognizable by enrichment of the heaviest and lightest isotopes of xenon, (2) *s*-process (slow thermonuclear neutron addition in red giant stars) xenon, enriched in middle xenon isotopes. This component may also be related to a component enriched in $^{13}\text{C}/^{12}\text{C}$ by a factor twice that of terrestrial⁵, and (3) Ne-E, which is essentially pure ^{22}Ne , ostensibly derived from monoisotopic ^{22}Na decay and possibly produced in a supernova.

Interpretation of the noble gas anomalies has been one of the most controversial subjects in cosmochemistry for more than a decade, since no diagnostic test has been available which uniquely identifies the astrophysical process(es) responsible for these exotic components. The reported observation that 'fission' xenon is chemically associated with anomalous nitrogen (-326‰) suggests a nucleosynthetic origin for the two, such as production in a supernova rather than fission of a superheavy element, particularly since fission-produced components of Ba, Nd and Sm are absent in these phases as shown by other recent work⁶. A major remaining obstacle is the apparent lack of a nucleosynthetic environment capable of co-producing the xenon and nitrogen isotopic anomalies. A nucleosynthetic process which produces the observed effects is not yet known.

Another tantalizing possibility raised by the report is the possible association of

heavy nitrogen ($+153\text{‰}$) released in the final high temperature steps of bulk Murchison (but absent or masked in the acid residues) with the enormous ^{13}C enrichment of $+1100$ per million observed in the high temperature release steps of Murchison acid residues⁵. Such a relation may suggest a novae ^{15}N origin and have the added advantage of simultaneous production of Ne-E via ^{22}Na . It still remains to be shown, however, whether the heavy nitrogen and carbon isotopic components are related, but presently unobserved, due to experimental procedures, or whether it is simply that they are not co-produced. If the latter is the case, then a greater variety of astrophysical processes are required. There are many other possible candidates in the literature, including local production, and future isotopic correlations will be needed to deduce the nature of the events responsible for the genesis of these anomalies.

The striking isotopic anomalies in car-

bon, nitrogen and noble gases potentially provide a glimpse into the very earliest history of the Solar System and possibly into stellar nucleosynthesis as well. The observed associated isotopic anomalies have presented some exciting possibilities for early solar and presolar system processes. Further resolution of the extent and nature of correlation for these exotic meteoritic isotopic species will provide a more extensive view of the complex processes operating in the early Solar System.

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Nitrogen fixation

A zoocoenotic symbiosis?

from John Postgate

SYMBIOTIC nitrogen fixation involving plants is a familiar process, fundamental to the continued stability of the biosphere. It is widespread throughout the plant kingdom, and a persistent question has been whether comparable symbioses occur with animals, particularly with those who habitually consume low-nitrogen food, such as wood.

Waterbury, Calloway and Turner¹ have now isolated a novel halophilic bacterium capable of microaerobic nitrogen fixation from a gland linked to the oesophagus of six species of shipworm, a wood-eating bivalve mollusc. As the bacterium can both digest cellulose and fix nitrogen they suggest that its presence may explain how these shipworms are able to use wood as their main source of food.

Positive reports of associations between nitrogen-fixing bacterium and animals go back at least as far as a suggestion made in 1933 and now discounted, that such bacteria are present in the blood of whales (and protect their hosts from the bends) and proposed hosts include sea urchins, sea squirts colonized by cyanobacteria, termites, other wood-eating invertebrates, guinea pigs, ruminant mammals and even man (see ref. 2 for a review). Indeed, a 'nitrogen-fixing' goat, which lives entirely on waste paper, has been a genetic engineer's half-joke for at least a decade. Diazotrophic bacteria normally inhabit parts of the intestinal tracts of termites, pigs, ruminants and man but their diazotrophic activity is generally low and their contribution to the nitrogen economy of their host seems slight (see also ref. 3).

Moreover, such zoocoenotic assoc-

iations seem to be rather casual and to lack the specificity found with most of the well-established plant symbioses, in which a specialized microbe will colonize but a limited range of plant hosts.

The bacterium discovered by Calloway and Turner seems to be more than just another casual commensalism, because the gland in which it is found, the gland of Deshayes, is directly involved in cellulose digestion by the bivalve. The bacteria colonize this gland specifically, in large numbers, apparently as a pure culture. And they are also cellulolytic bacteria. Thus the authors proposed that they not only provide the cellulases to enable their molluscan host to digest its normal diet, but that they also provide sufficient fixed nitrogen to balance their host's nitrogen budget.

Quantitative studies with $^{15}\text{N}_2$ are now needed urgently, because the contribution of the bacteria to the nitrogen economy of the bivalve may yet be quantitatively as trivial as it is in the rumina of sheep and cattle. The evidence for specificity of both the microbe and its location, however, together with both cellulolysis and diazotrophy, is most intriguing.

To biotechnologists the report has a further twist: this microbe (still unnamed) is the first to be reported that is capable of cellulolysis and diazotrophy together. Most natural cellulolytic systems (for example, those decomposing straw) involve communities of microbes: fungi and sometimes bacteria breaking down the cellulose, diazotrophs (clostridia, enterobacteria, azospirilla or azotobacters) using the products for nitrogen fixation, often

with non-specific bacteria helping to sustain a low oxygen regime. An organism which combines the first two activities in one cell could have practical advantages, a point which, according to Waterbury and colleagues has not escaped the attention of 'genetic engineers'. To construct a nitrogen-fixing cellulolytic bacterium by genetic manipulation ought not to be diffi-

cult, but this particular race seems to have been won by the microbial ecologists! □

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History of science

Scientific instruments and history

from David W. Hughes

THE abundance of excellent papers presented at the Third Scientific Instrument Symposium* made it plain that scientists should think much more positively about the museum potentiality of their old instruments and about keeping careful documentation of their instruments' lives.

Between the laboratory and the museum there looms a gap of about 50 years — a chasm which is difficult to bridge. We should all try to make the road to the museum easier. In many schools, colleges, universities and laboratories, where science was, and still is, taught and research prospers, there is probably a dusty cupboard hiding a treasure store of scientific instruments that would gladden the heart and mind of many a scientific historian. Think twice before throwing old instruments away!

Speakers at the symposium came from museums all over Europe and took many different approaches to the subject. One approach was to look at the work of particular individuals. G. Hartl (Deutsches Museum, Munich) introduced G.F. Brander (1713-1783), one of the most important scientific instrument makers in Germany during the eighteenth century. Brander could produce glass scales on which the millimeter was divided into 30 equal parts; a considerable advance in the accuracy of measurement was thus generated. By 1780 the catalogue of Brander's workshop included 102 instruments covering the main areas of contemporary natural science: astronomy, mathematics, optics, meteorology, geodesy and physics. Brander's work was on show at the museum; a superb exhibition of the instruments from the workshop has been assembled by a team of Deutsches Museum scientists under the leadership of Alto Brachner and will stay on display there for the foreseeable future.

Another Munich personality was Joseph von Fraunhofer (1787-1826). It was through Fraunhofer's work that the scientific and industrial basis for the production of pure, defect free optical glass and its manufacture into lenses, achromatic lens

combinations and prisms was created. The desire for a more accurate determination of the refractive indices of these glasses led to the discovery of the dark lines in the solar spectrum that bear his name. The small glass works at the Monastery of Benediktbeuern where Fraunhofer manufactured glass between 1807 and 1819 still exists.

The teaching aspects of scientific instrumentation were emphasised by A.D. Morrison-Low (Royal Scottish Museum, Edinburgh), who has investigated how J.H. Bennett introduced microscopy into the teaching curriculum for medicine at the University of Edinburgh in 1841. The work of Bennett and his colleagues helped transform the microscope from a scientific toy into a first-rate research instrument.

Scientific apparatus can also be studied by concentrating on a specific task. R.G.W. Anderson (Science Museum, London) took distillation as his example, stimulated by the Science Museum's recent acquisition of some important tenth to twelfth century chemical glassware of Islamic origin. He has been able to trace the development of alembics and retorts, cucurbits and receivers.

Not surprisingly, museums featured strongly at the Symposium. G. Fodera Serio discussed a museum that may be opened at the Palermo Astronomical Observatory, Sicily. In 1790 Guiseppe Piazzi, the first director, equipped the observatory with the most up-to-date instruments he could get. Many were

obtained from the famous London instrument maker Jesse Ramsden, the two largest being a five-foot alt-azimuth circle and a transit instrument. These have been lovingly restored and provide a beautiful 'snapshot' of an observatory of that time. Another new display was described by Hemming Anderson (Soro Akademi, Denmark). For the last 80 years the physical instruments collected by Adam Wilhelm Hauch have been stored away in packing cases. The instruments of French, English, Dutch and Danish manufacture are now restored to view. A unique discovery was described by A. Brachner (Deutsches Museum). In December 1982 he was contacted by the Benedictine Monastery of Ochsenhausen, Bavaria and asked for advice about the conservation of an astronomical instrument. On going to the monastery he found their baroque observatory dome contained a beautiful example of a late 18th century, three-metre radius, azimuthal quadrant that had probably been used for teaching purposes.

Scientific expeditions are a potential source of information for the historian of scientific instruments. In 1878 the Dutch Schooner *Willem Barents* sailed to the Spitsbergen and Barents Seas. Systematic scientific observations, concentrating on cartography, maritime meteorology, physical oceanography, and terrestrial magnetism, were carried out. W.F.J. Mörzner-Bruyns (Maritime Museum, Amsterdam) has been able to reconstruct the inventory of instruments used on board the ship.

Thomas Graham wrote in 1838 'It is curious how much the progress of science depends upon the invention and improvement of instruments.' It is obvious from the Symposium that the study of old scientific instruments and methods is in capable hands. Many modern scientists would be fascinated by the genealogy of their instruments and can play an important role in saving our scientific heritage by helping instruments find their way to museums. □

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100 years ago

ON Monday, September 24, about 9 p.m., a remarkable phenomenon occurred at Käringsön, in the province of Bohus, Sweden. During a perfect calm a violent whirlwind suddenly arose from the south-east, carrying with it a quantity of sand, earth, and straw, when suddenly a bright light lit up every object and made the night as clear as day. This was caused by a magnificent meteor, egg-shaped in form, which appeared in the zenith, and which at first seemed to consist of myriads of large sparks, gradually changing into a star shining with a blinding lustre, and which burst, with all the colours of the rainbow, in the north-west, four to five metres above the horizon. When the meteor had disappeared the wind suddenly fell, and it was again perfectly calm. The phenomenon lasted

about sixty seconds. The wind had throughout the day been south and very slight.

A sharp shock of earthquake was felt at Bermuda on October 20, but no damage was done. A shock was felt at Tashkend at twenty minutes past two on the morning of the 27th, accompanied by loud subterranean ramblings. A despatch from Smyrna dated October 28 reports that the wall surrounding the town, the Aqueduct, and the Hadji Hussein Mosque have been damaged by an earthquake. The minaret and dome of the Hadji Ali Mosque at Capan Vourla have also been injured. At the last-named town one hundred and sixty-nine persons have been seriously, and sixty-one slightly, hurt. Seventy-nine wounded people are in the hospitals.

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Parallel visual computation

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The functional abilities and parallel architecture of the human visual system are a rich source of ideas about visual processing. Any visual task that we can perform quickly and effortlessly is likely to have a computational solution using a parallel algorithm. Recently, several such parallel algorithms have been found that exploit information implicit in an image to compute intrinsic properties of surfaces, such as surface orientation, reflectance and depth. These algorithms require a computational architecture that has similarities to that of visual cortex in primates.

UNTIL recently, the profound difficulty of vision was not fully appreciated, in part because we humans are so good at seeing. We can recognize objects in images very fast—within a few hundred milliseconds—and without appreciable effort. It therefore came as a surprise in the 1960s and the early 1970s when pioneering workers discovered that automated image interpretation requires an enormous amount of computation and that it is very hard to find simple features in real images that allow objects to be separated from one another and recognized^{1,2}.

Recent work in computer vision has been strongly influenced by two factors. The first is an increased awareness of the architecture of biological visual systems combined with a realization that parallel processing may be essential for competent vision systems because the serial architecture of conventional digital computers is too inefficient to deliver the massive amount of computation required³⁻⁷. The second factor is a much better understanding of how the domain of two-dimensional intensity arrays is related to the domain of three-dimensional objects. In an image of an object, each element in the intensity array depends on the illumination and on the 'intrinsic' properties of the three-dimensional surface patch being imaged, such as its orientation relative to the viewer, its orientation relative to light sources, and its reflectance. When these optical constraints are combined with plausible assumptions about the nature of physical surfaces, it is possible to interpret the information that is encrypted in an intensity array in terms of intrinsic properties of three-dimensional surfaces⁸⁻¹².

This review discusses a parallel approach to visual computation and its relevance to visual processing in the cerebral cortex of primates.

Parallel architectures

The computational problems in vision are best understood at the early stage of visual processing where the inputs and the goal of the computation are known. To implement a computational theory, both the representation of the information and a well defined algorithm are needed. The hardware of a machine strongly affects the degree of difficulty in implementing and running an algorithm. The examples presented here were run on conventional digital computers simulating parallel machines, because the flexibility of a general-purpose computer was useful in exploring a variety of parallel architectures without making a commitment to any particular one.

Conventional digital computers use von Neumann architecture in which a single central processor performs sequential operations on data structures (including programs) stored in a general-purpose memory. Although central processors are very fast today, their sequentiality seriously limits the rate at which computations can be performed. The dramatic decrease in the cost of logic circuits has made parallel architectures attractive if the problem can be solved with a parallel algorithm.

In a parallel computer many separate processing units operate in parallel and exchange information through a communication network. The parallel architectures that are now being studied can be broadly classified according to the computational power of the processing units and the type of information that is exchanged between them^{13,14}. The most complex, and the most difficult to analyse, is 'message-passing' parallelism, in which symbolic messages are passed between units that are as powerful as von Neumann machines¹⁵.

A much simpler type of parallel architecture is based on processing units that exchange only approximate real values and can perform only simple arithmetic using limited memory. Models of neural networks are frequently of this 'value-passing' type, with the average firing rate of a neurone interpreted as the transmitted value. The power of this architecture lies in its ability to propagate information simultaneously along private links between units. However, the required connectivity and the way the transmitted values are used are different for each problem, so the flexibility of a general-purpose computer is lost and a formidable wiring problem is created. In this review we explore how value-passing parallelism can be applied in vision.

Relaxation

The goal of many problems in vision is to find the optimal interpretation of an image consistent with known optical and geometrical constraints. The difficulty is that an enormous number of constraints must be simultaneously satisfied. One way to solve such large optimization problems is to implement the constraints as excitatory and inhibitory links between processing units and to allow the units, whose values represent the physical properties of objects, to iteratively approach a self-consistent solution¹⁶⁻²³.

The classical problem of finding the shape of a soap film bounded by a non-planar wire hoop illustrates how a global solution can be achieved through successive local interactions. Imagine that the soap film is viewed from above and that its height is represented by a number in each cell of a two-dimensional array. The wire hoop fixes the heights around the edge, but an interior height is only constrained by being equal to the average of its neighbours. Regardless of the initial assignment of the interior heights, the correct shape of the soap film can be computed by iteratively assigning to each interior cell the average of its neighbours, a process called relaxation.

Just as the balance of forces acting on a piece of soap film act as physical constraints on the solution, so the optical constraints of image formation and the general nature of the surfaces causing the image restrict the plausible solutions that are consistent with the intensity array. The boundary condition for the computation is the raw input—that is, the whole intensity array—just as the height of the wire hoop is the boundary condition for the soap-film problem. The soap-film analogy is

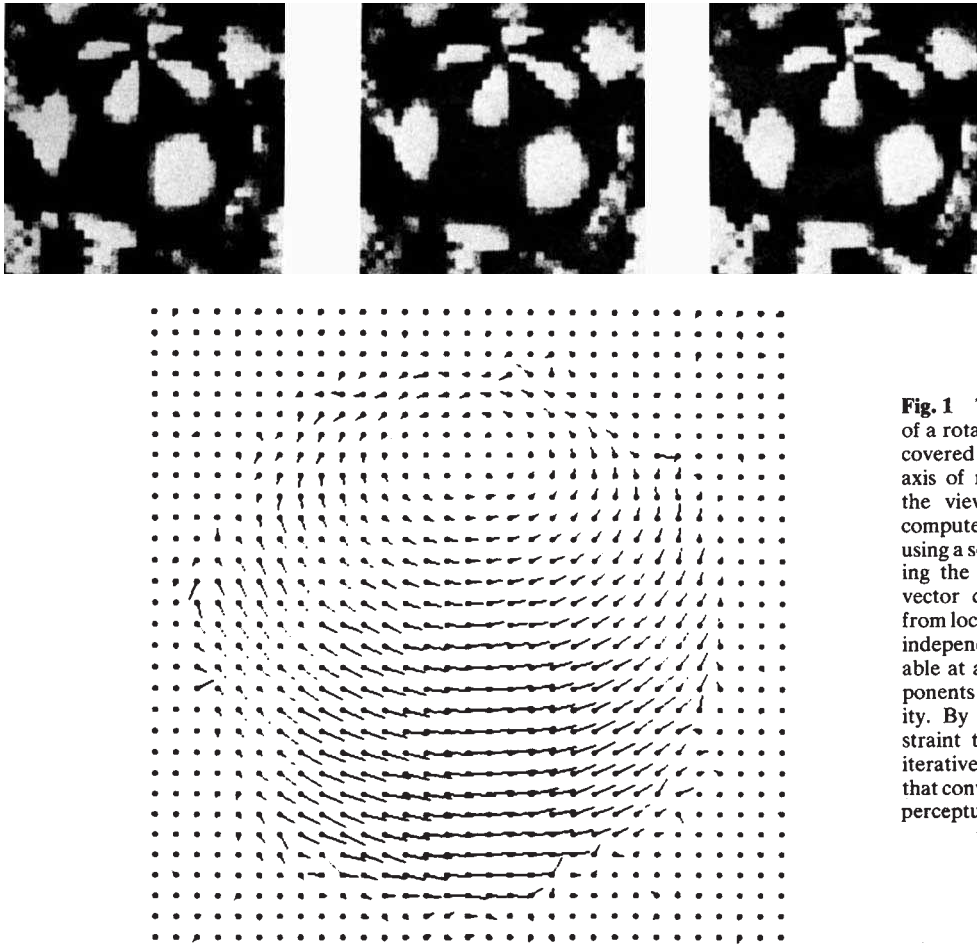


Fig. 1 Top, three successive frames of a rotating sphere whose surface is covered with a pattern and whose axis of rotation is inclined towards the viewer. Bottom, flow pattern computed by a relaxation algorithm using a sequence of 32 frames including the above three. Each velocity vector cannot be computed solely from local information since only one independent measurement is available at a given point, and two components are needed to specify velocity. By adding the additional constraint that the flow is smooth, an iterative algorithm can be derived that converges robustly to the correct perceptual interpretation²⁶. (Courtesy B. K. P. Horn.)

helpful in conveying the general idea behind value-passing computation, but as we shall see, parallel visual computation can differ from the soap-film example in important ways.

Surface orientation

The goal of early visual processing is to recover intrinsic properties like surface orientation^{6,24}. Such properties cannot be deduced from the local intensities alone because there are infinitely many possible combinations of orientation, reflectance and lighting that could explain any single intensity value. So, in addition to the optical constraints that relate image intensities to combinations of intrinsic parameters, the interpretation process must also use plausible assumptions about the world that constrain the intrinsic parameters at neighbouring locations. For example, the orientation and reflectance of a surface at one point strongly affect its *probable* orientation and reflectance at neighbouring points, though they do not definitely rule out any of the possibilities.

Because it must use constraints that are plausible but not definitive, the interpretation process in early vision has the form of a massive best-fit search whose objective is to find sets of intrinsic parameters that are compatible with the optical constraints and satisfy as well as possible the plausible assumptions that link intrinsic parameters at neighbouring points.

Horn's analysis of the recovery of surface orientation from the shading in an image is one of the earliest examples of how the physics of imaging can constrain the three-dimensional interpretation of an image^{24,25}. If the reflectance function of the surface and the direction of illumination are known, then an iterative 'shape from shading' algorithm can fill in the most likely surface when given the surface shape along the boundary of the object (Fig. 2). The result of the computation is an intrinsic image; that is, a map of an intrinsic property (in this case surface orientation) in register with the image⁶. The same type of algorithm can also be used to compute the intrinsic image of local velocity²⁶, as shown in Fig. 1.

Stereo fusion

In addition to being able to recover the surface orientation from the shading of a bounded surface, the human visual system can recover the relative depth of surface markings by fusing binocular images. Julesz has demonstrated that when each eye is presented with the same pattern of random dots, except that one region in one eye is horizontally displaced, then this displaced region is perceived as a surface with a different depth from its surround²⁷. Because all the dots look the same, it is difficult to decide which dot in one image corresponds to which dot in the other image, but this correspondence must be decided to perceive depth.

In the Marr-Poggio cooperative algorithm for stereopsis, each unit stands for a hypothesis about the correspondence of a particular pair of dots, and it therefore represents a patch of surface at a particular depth²⁸. There are excitatory interactions between neighbouring units with the same depth to ensure continuity of surface, and inhibitory interactions between units that represent different depths at the same image location to ensure that depth assignments are unique; if the sum of all the inputs to a unit from the two images and from local interactions is above a threshold, the value of the unit is set to 1, and otherwise to 0. During the relaxation various combinations of depth assignments are tried and the network eventually 'locks' into a consistent solution in a way that resembles our perceptual experience when we fuse a random-dot stereogram²⁷.

There are important qualitative differences between the shape-from-shading problem and stereo fusion. Like the soap-film problem, the shape-from-shading algorithm searches a space that has the character of a single hill because each step brings the system closer to a unique solution, so that convergence is guaranteed. In contrast, the stereo-fusion algorithm has a search space that resembles a mountain range: even if the search can be made to climb towards progressively better interpretations, the final solution may be only a local optimum rather

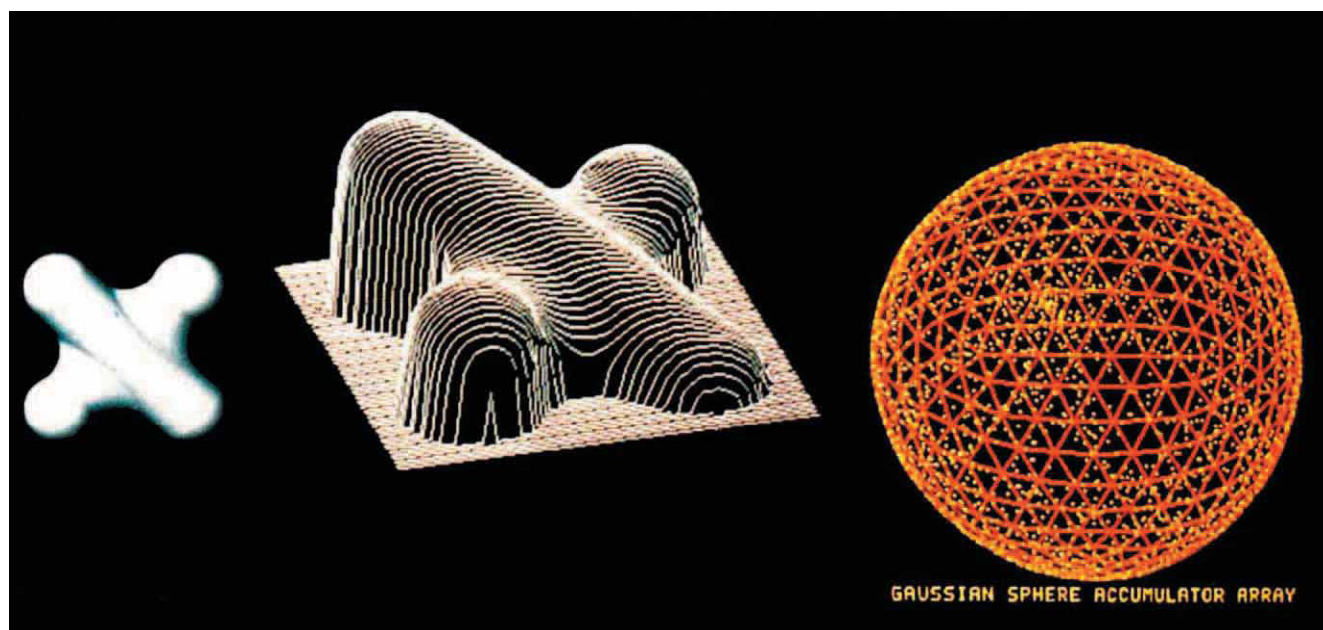


Fig. 2 Demonstration of an algorithm that generates both the shape of an object and the direction of illumination given only a shaded image of the object. The input to the algorithm is the image shown on the left. The output of a relaxation algorithm that was used to compute the shape is shown in the centre. During the computation the current estimate of the surface normals was used to compute an estimate for the direction of illumination in a two-dimensional space of direction units (right). The direction with highest activation in turn was used to obtain a better estimate of the surface normals, and the process was repeated until the estimates converged⁶⁰. (Courtesy C. M. Brown.)

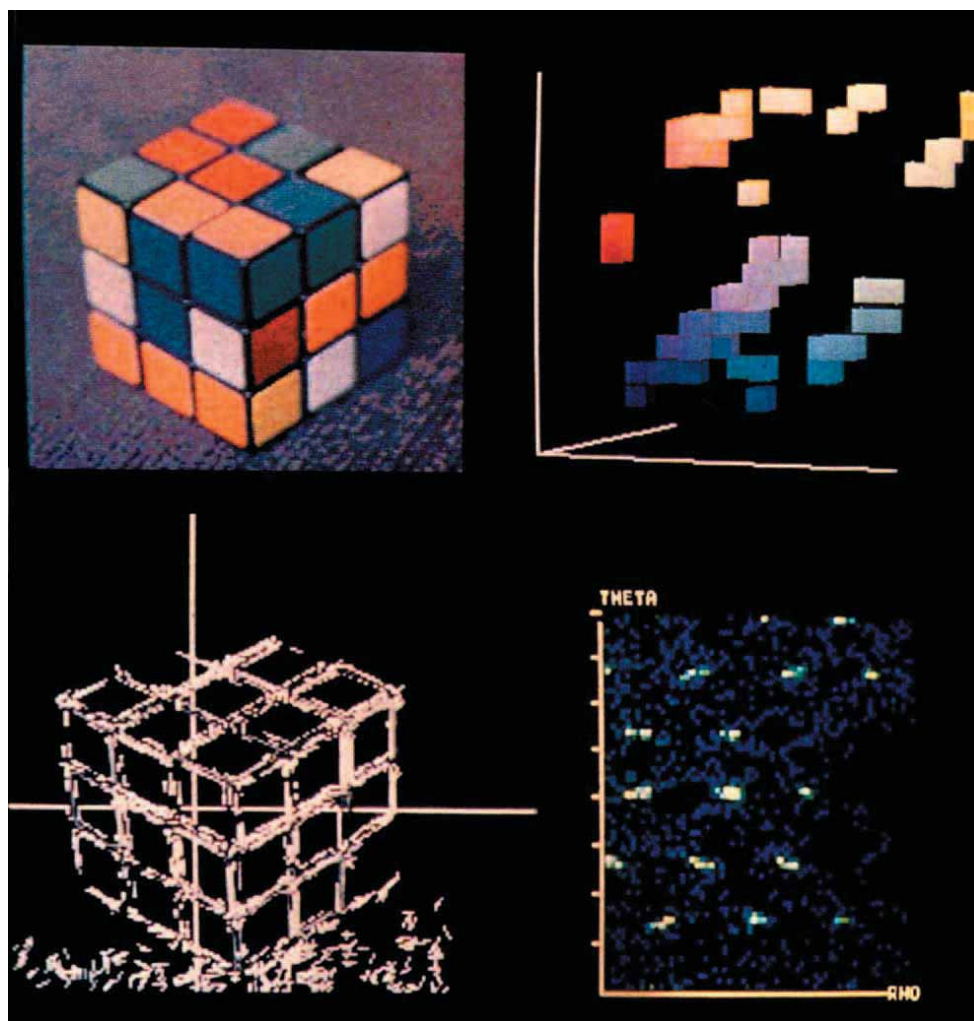


Fig. 3 The image of a Rubik cube (top left) mapped into two global feature spaces: colour space (top right) and line space (bottom right). In the colour space, a transformation was performed from each point in the colour image to a three-dimensional space of units whose axes represent three colour components. The colour of each unit is the colour it represents, and only the units with the highest activity levels are shown. Clusters of units are activated by all the faces of the Rubik cube with roughly the same colour value. Note, however, that the textured background activates many units in colour space. In the line space, local edges were first detected in the image by finding the position where the intensity was changing the fastest (bottom left). Note that many of the edges are incomplete and other edges are obscured by noise in the background. Long lines in the image can be detected by allowing colinear edges to support each other. This may be done by using another two-dimensional space (line space) in which each point represents an infinite line parameterized by the line's perpendicular distance from the origin (ρ) and the angle with the x -axis made by the perpendicular to the line from the origin (θ). Each local edge unit provides input to all the global line units in line space with which it is consistent. Edge units may be inaccurate and incomplete, but lines in the image appear in line space as the units with the highest activation (white clusters)^{58,67}.

than a global one. This difference in the character of the search space is partly due to the fact that stereo fusion involves discrete choices between alternative correspondences for each dot, whereas the shape-from-shading computation involves gradual changes of continuous parameters, though in general there may be local optima even with continuous parameters.

The relative simplicity of the search space for the shape-from-shading algorithm disappears if it is generalized to cases where the locations of object boundaries are not known in advance and can only be decided by using assumptions about the smoothness of surfaces. In the more general problem, choices of values for continuous variables like the local surface orientation must interact with choices of values for discrete variables like the presence or absence of an edge. Search spaces that contain many local optima may well be unavoidable when designing algorithms that make both kinds of choices.

A search procedure based on statistical mechanics has recently been introduced that can escape from the local optima at which simple hill-climbing algorithms would be trapped^{29,30}. If noise is added to the decision rule, so that each unit sometimes adopts a state which is less consistent with the current states of the other units, the system relaxes to different global configurations with different probabilities. After sufficient time the probability of finding it in a global configuration depends only on how consistent that configuration is, with more consistent configurations having higher probabilities. This kind of search is effective in situations in which the correct interpretation is much more consistent than any other, and this may well be typical of normal vision³¹.

Representations

The performance of an algorithm depends on what information in the image is explicitly represented by the units and how that information is coded. In the Marr-Poggio algorithm the depth of a surface patch is represented by activating one of a set of units, each of which is 'tuned' to a different depth. This type of representation is qualitatively different from an analog representation in which the firing rate of a single unit directly encodes the value of a physical variable.

By dividing the range of a continuous variable into discrete intervals, each unit becomes, in effect, a hypothesis about the existence of a feature in the image, and the firing rate can then be used to encode the probability of the hypothesis being correct rather than the value of the physical parameter^{32,33}. This type of 'interval encoding' makes it easier to implement searches in spaces that contain both continuous and discrete variables, because it treats continuous variables in the same way as discrete ones.

Several variables in the visual cortex are represented by broadly-tuned neural mosaics that seem to be a form of multi-dimensional interval encoding (Fig. 4). For example, disparity-sensitive neurones in primate visual cortex fall into three groups: those that are tuned to the plane of focus and those that respond to visual stimuli that are either convergent or divergent³⁴. The same neurones also respond preferentially to oriented edges and line segments; these are broadly tuned over 20–40° and their range of sensitivities overlap considerably^{35,36}. The broadness of the tuning need not imply imprecision, since we have, for example, only three types of cone photoreceptors with broadly-tuned spectral sensitivities, but we are nonetheless capable of fine colour discrimination.

An important practical issue that arises when implementing a relaxation algorithm is the spatial resolution of the units that represent intrinsic properties. With high resolution, fine irregularities may introduce noise, but with low resolution important discontinuities may be smoothed out. Experience has shown that having a range of resolutions is most effective^{37–40}, and experiments indicate that a range of resolutions is also used in the nervous system^{35,41,42}. For example, in 1979 Marr and Poggio proposed an improved model of stereopsis that incorporated multiple spatial resolutions^{43,44}. Matching between the right and left images was first performed on the largest channel, which

has the least ambiguity, and this helped matches at progressively finer scales. The issue of exactly what primitive features should be used to perform the matching is not yet resolved^{45–47}.

Visual maps and feature spaces

Our visual system is capable of computing surface orientation from shading and depth from binocular images, but we do not yet know how these computations are organized. We do know that an important organizing principle in the early stages of visual processing is the orderly mapping and remapping of the visual field. Twelve maps of the visual field have been found in the primate cerebral cortex so far^{48,49}, and there are also many subcortical maps.

In retinotopic maps, neighbourhood relationships between nearby positions of the visual field are retained, while through convergence and local interactions the response properties of cells are modified. Already at the level of a retinal ganglion cell, light falling on surrounding positions inhibits the central response, a combination that is well known to enhance contrast information⁵⁰. In cortical maps higher-order combinations are used to compute orientation and directional selectivity³⁶. Extraction of these features has proven to be very effective in preprocessing images for computer vision.

Most of the neurones in extrastriate visual maps receive their major visual input either directly or indirectly from the striate cortex, the first area of cerebral cortex to receive visual input, and they have larger receptive fields and less topographic order than neurones in striate. The proportion of cells sensitive to a particular property is different in the various maps. For example, the cells in an area called MT appear to be particularly sensitive to the direction of stimulus motion^{51–53} and their responses are selectively influenced by motion of the background⁵⁴ (Fig. 5). In the inferior temporal cortex the receptive fields of some neurones cover most of the visual field and their preferred stimulus is often not an oriented edge^{55,69}. The functions of most of these visual maps remain obscure⁵⁶.

Parts of an object can often be identified as a whole by common properties, such as colinearity of edges, colour and direction of movement, even though the object is partially obscured by other objects. Barlow⁵⁷ has pointed out that an economical way to link up parts of an image having a common feature is to collect that information in a single location irrespective of where it came from in the image. Neighbouring locations in this new space represent neighbouring values along a non-spatial dimension rather than neighbouring positions in the image; two examples of such non-retinotopic feature spaces are shown in Fig. 3.

The first use of a transformation from an image to a non-retinotopic feature space was made by Hough⁵⁸, who patented a machine based on this principle in 1962. Hough found that noisy particle tracks in bubble chamber photographs could be efficiently analysed by transforming the data into a line space in which each point represented an infinite line with a particular slope and perpendicular distance from the origin, as explained in Fig. 3. Each detected edge 'votes' for all the lines in line-space that are consistent with it. Even though each piece of evidence is noisy, a line that is present in an image will receive many votes.

Hough transforms have been generalized in several ways. First, the feature space need not be simply related to the local features computed in intrinsic images⁵⁹; second, the units in the feature space may themselves interact and even feed back to the intrinsic images⁶⁰. For example, in the computation of surface orientation from shading, the direction of the light source had to be specified as a boundary condition. The two angles that specify the direction of illumination are global variables and can be computed from the intensity array and from a very approximate intrinsic image of the surface orientation. The new direction of illumination can then be used to refine the intrinsic image. The coupled algorithms converge to an intrinsic image of the object's surface and the direction of the light source⁶⁰, as shown in Fig. 2.

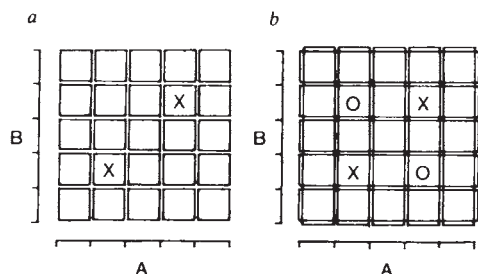


Fig. 4 Schematic drawings of two ways that a two-dimensional space of features (such as orientation angle and disparity) can be represented by populations of units. *a*, Each unit is assigned to a tile of the mosaic and jointly represents the intervals given by its position in the mosaic. *b*, Each unit is assigned to an interval of one variable and is insensitive to the other. If the features of two different objects are simultaneously present, then in representation *a* two units are activated, but in representation *b* four units are activated, as indicated by the Xs. Note that in the latter case there is an ambiguity in which pair of features go together since the same units would also be activated by 'ghost' objects whose features are indicated by the Os.

Although extrastriate maps have an overall hierarchical organization, forward projections are generally accompanied by reciprocal feedback connections. The function of these feedback connections is unknown, but one possibility is to enhance or 'focus attention' on the parts of the image having a common feature⁵⁷. A more general function of feedback, suggested by Fig. 2, is to link two cortical areas for the purpose of joint interdependent computation.

Neural representations

The map of surface orientation is an important intermediate step in building a three-dimensional interpretation of an image. Evidence has not yet been found for neurones in the visual cortex sensitive to surface orientation, but the appropriate stimuli have probably not yet been used to look for them. This is not an easy task given the variety of cues that could be used to generate images of surfaces and the large number of visual areas. Although computer vision may offer suggestive hints, it should be emphasized that algorithms are not unique solutions to problems; the example of stereopsis, for which we have several working models, illustrates that further evidence from psychophysics as well as physiology will be necessary to constrain possible explanations^{22,47}.

Enough neurones exist in the visual cortex to represent explicitly many features of natural images to which the human visual system is sensitive³¹. Beneath every square millimetre of cortex there are $\sim 10^5$ neurones; in the striate cortex this is about the size of a hypercolumn that contains all the machinery needed to analyse a single patch the size of one receptive field⁴². Most neurones in the visual cortex respond best only when two or more features are simultaneously present. Even though the number of neurones in the visual cortex seems comfortably large, there is a finite limit to the number of features that can be explicitly represented, and it is particularly costly to represent all possible conjunctions of properties. For example, if enough neurones were provided to represent all possible combinations of values of edge orientation, disparity, velocity, spatial frequency, colour, etc., the number required would increase exponentially with the number of dimensions. With a resolution of 10 intervals on each dimension, the 10^5 neurones in a hypercolumn could only cover a five-dimensional feature space⁶⁸.

One solution to the exponential explosion is to give up on making all conjunctions explicit and to represent different features in different maps⁵⁹. The price of this compromise is a possible confusion in the correspondence between pairs of features in different maps that are simultaneously activated by several objects (Fig. 4). In fact, when coloured letters are briefly presented to human observers, incorrect matches between the colours and the letters are quite often reported in certain conditions, a phenomenon called illusory conjunctions⁶¹.

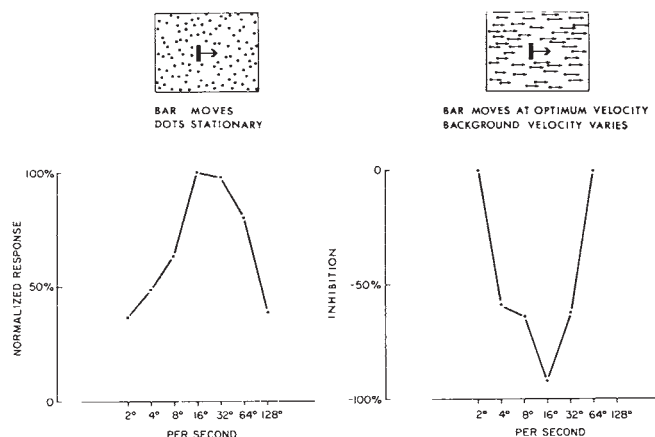


Fig. 5 Selective influence of a moving background of random dots on the response of a directionally-selective neurone in area MT of the owl monkey. The maximum firing rate occurs when a bar is moving in its preferred direction at its optimum velocity⁵⁴. Left, firing rate (normalized to the maximum rate) to a bar moving against a stationary background as a function of velocity. Right, response of the bar moving at its optimum velocity as a function of the velocity of the background. The response is inhibited if the background moves in the same direction as the bar with the maximum inhibition occurring when the background moves at the same velocity as the bar. The area of the visual field influencing the response to the bar was about 20,000 square degrees, 60 times the area of the classical receptive field. In V4, another area of extrastriate cortex, the responses of some cells to coloured stimuli are strongly influenced by the colour of the surrounding illumination^{70,71}, and these long-range effects may be mediated by a recently identified colour pathway originating in striate cortex⁷². The discovery that the surround is as selectively tuned as the classical receptive field may lead to a better understanding of the algorithms used in the visual system to compute intrinsic images such as that of optical flow and reflectance. (Courtesy J. M. Allman.)

Another way to cover a feature space of high dimension with a limited number of units is to assign them to large, overlapping regions. A single point in feature space is then represented not by activity in a single unit, but by the joint activity of many units. Hinton⁶² has recently shown that accurate information is not lost by these coarsely tuned units; the information has simply been distributed over a population of units. Moreover, far fewer units are required to achieve the same accuracy. For binary units that respond only when a feature falls within their region of feature space, the number of units required to achieve a given accuracy is proportional to $1/r^{(k-1)}$, where r is the radius of the region and k is the dimensionality of the space. Thus, the relative efficiency of coarse coding increases with the dimensionality of the space being covered.

Although coarse coding efficiently encodes single features, two nearby points in feature space that are simultaneously excited may be confused. The sparseness of the features that occur in any one image therefore sets an upper bound on the allowable coarseness of the coding.

Future directions

It is generally thought that at the later stages of visual processing the features of an object are represented relative to a frame of reference based on the object rather than in a frame relative to the retina¹⁰. By imposing an appropriate frame of reference on an object, and recoding features relative to this frame, the visual system can generate 'object-based' features that are independent of viewpoint and therefore represent the invariant shape of the object. Algorithms that use generalized Hough transforms in coarsely coded feature spaces^{63,64} have been proposed for choosing the object-based frame and generating the object-based features. Thus, the same type of parallel relaxation algorithm that was used in early visual processing might also be useful for higher levels. This would be consistent with the similar cytoarchitectonic organization found throughout the cerebral cortex, from primary sensory to higher associational areas^{65,66}.

Despite the power of parallel computation, some problems in vision clearly require sequentiality. Consider, for example, the role of eye movements in building up a detailed internal representation of a complex object, such as a face. The integration of sequentially acquired information raises issues about the nature of visual working memory and the central control of processing. Parallel models of these aspects of vision are being explored but are beyond the scope of this review.

We have described a recent trend within computer vision

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towards parallel models and the relationship of these models to processing in visual cortex. As the parallel architectures in computer vision come to resemble biological visual systems more and more closely and as we gain more experience with these architectures, we may achieve new insights that will better help us to appreciate nature's designs.

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ARTICLES

Turbulent dissipation and shear in thermohaline intrusions

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Laterally coherent patches of turbulence were discovered on the upper and lower boundaries of thermohaline intrusions. The ratio ϵ/J_b is used to compare the relative importance of turbulence produced by Reynolds stress and that produced by the buoyancy flux of double diffusion. Velocity profiles show near-inertial motions through the intrusions.

FRONTAL regions in the ocean are characterized by the frequent occurrence of 5–30 m thick interleaving lenses of water, which are referred to as intrusions. The horizontal and vertical transports of heat and salt by intrusions may be major factors in the flux balances of fronts. Therefore, determination of the mechanisms by which the intrusions are formed and the rates of subsequent mixing is essential to understand frontal dynamics.

During the past year, we have used the Advanced Microstructure Profiler (AMP)¹ to take many sets of closely spaced profiles through intrusions in the Bahamas, in a warm-core Gulf Stream ring, and in the California Current. Velocity microstructure measurements were used to determine ϵ , the rate of viscous dissipation of turbulent kinetic energy. Observations of temperature–salinity (T_s) fine structure were used to estimate J_b , the buoyancy flux across interfaces believed to be sites of double

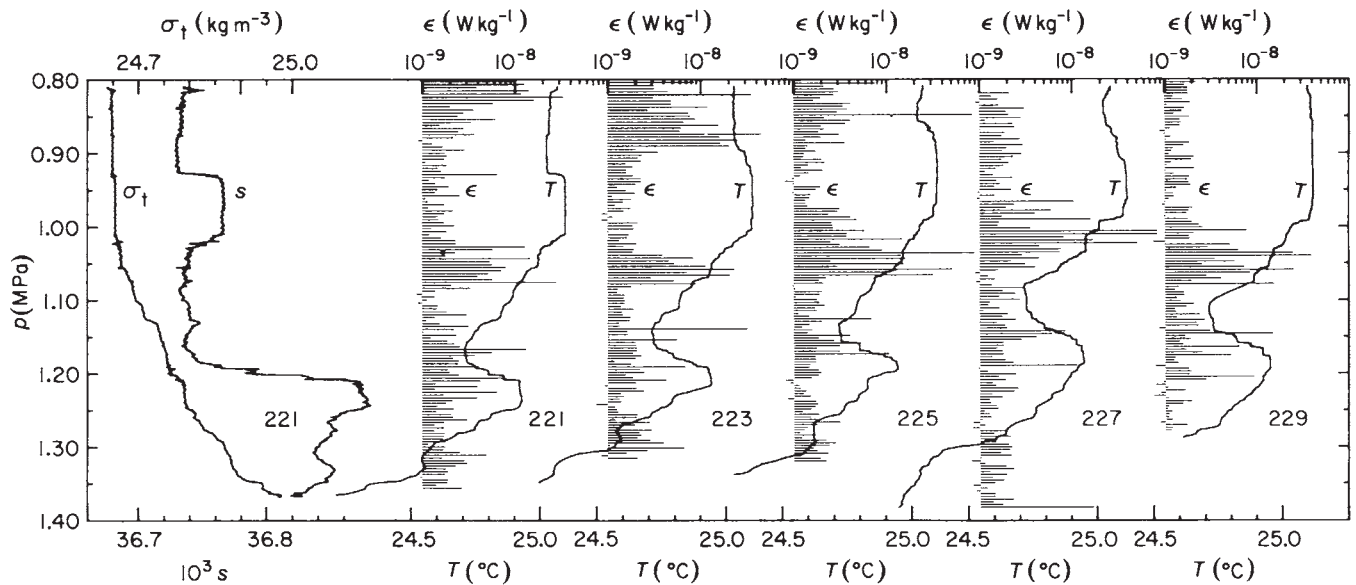


Fig. 1 A sequence of five profiles taken in the Bahamas. During the 45-min observation period, the ship drift was nearly linear. The average separation was 240 m. A pressure of 1 MPa corresponds to a depth of 100 m. The panel on the left contains salinity (s) and $\sigma_t = \rho(s, T, 0)$ for AMP 221. Other panels contain temperature (T) and $1/2$ m averages of ϵ , which were computed making standard assumptions about isotropy. There are three laterally coherent patches of elevated ϵ : (1) below the temperature maximum near 1 MPa, $\bar{\epsilon} = (4.8\text{--}8.9) \times 10^{-9} \text{ W kg}^{-1}$; (2) above the temperature maximum near 1.2 MPa, $\bar{\epsilon} = (1.2\text{--}2.6) \times 10^{-9} \text{ W kg}^{-1}$; (3) above the temperature maximum near 0.95 MPa in AMP 221, 223 and 225, $\bar{\epsilon} = (2.4\text{--}6.0) \times 10^{-9} \text{ W kg}^{-1}$.

diffusion. Examination of ϵ/J_b provides a quantitative estimate of the fraction of the observed mixing that is due to double diffusion. Velocity profiles, with a vertical resolution of 3 m, were also made through intrusions in the warm-core ring with Expendable Current Profilers (XCPs)². This report is based on an analysis of selected profiles from this extensive data set.

Double diffusion, buoyancy flux and turbulence

Intrusions usually appear as maxima or minima in both T and s profiles made in frontal regions. Well-defined stair steps of increasing temperature and salinity are often found above the Ts maxima and have been identified with the diffusive regime of double diffusion^{3,4}. Although regions below the Ts maxima are diffusively unstable to salt fingering, the second regime of double diffusion, the step structures that characterize salt fingering in other situations have been found on only a few intrusions⁵. However, the low static stability and intense thermal microstructure found below the Ts maxima indicate that vigorous mixing is taking place^{4,6,7}. Gregg and Cox pointed out that the microstructure activity could be due either to fingering or to shear-driven turbulent mixing produced by the spreading of the intrusions⁴. Subsequently, banded structures in optical shadowgraphs showed that salt fingers occur in intrusions^{8,9}, but there has not been a quantitative assessment of their contribution to the observed microstructure.

Both regimes of double diffusion produce positive (upward) buoyancy fluxes, $J_b \equiv -(g/\bar{\rho})\bar{u}_3'\bar{\rho}'$ where u_3' is the fluctuating component of the vertical velocity and ρ' is the fluctuating component of the density. J_b is one of the terms of the turbulent kinetic energy balance equation. In a simple shear flow this equation is¹⁰

$$\frac{\partial q^2}{\partial t} + \frac{\partial[u_1'(p'/\bar{\rho} + q^2)]}{\partial x_1} = -\bar{u}_3'u_1'\frac{\partial U_1}{\partial x_3} + J_b - \epsilon \left[\frac{\text{W}}{\text{kg}} \right] \quad (1)$$

where q^2 is the velocity variance and p is pressure. The terms on the left express the rate of change of the turbulent kinetic energy and the divergence of the turbulent transport, respectively. In many situations the turbulent intensity is steady and homogeneous, and these terms can be neglected. The first term on the right is the production of turbulent kinetic energy from the mean shear by the Reynolds stress.

A positive buoyancy flux imposed on the boundary of a fluid

layer produces turbulence within the layer. If the mean shear, $\partial U_1/\partial x_3$, is weak, so that the Reynolds stress is small, the balance of turbulent kinetic energy reduces to

$$J_b = \epsilon \quad (2)$$

If the layers are well-mixed and changing slowly with time, $\nabla \cdot J_b = 0$, then J_b and ϵ will be constant with height until the layers are terminated by a transition to a stable stratification¹¹.

We are not aware of any previous measurements, in ocean or laboratory, of the ϵ levels produced by double diffusion. However, the reasoning used for convection driven by heat must apply. Therefore, in steady, homogeneous situations where the Reynolds stress is negligible, an upper bound for ϵ is given by the sum of the buoyancy fluxes across the interfaces bounding the layer.

Expressions for the buoyancy fluxes are based on laboratory measurements of the rate of change of T and s across double diffusive interfaces¹². For salt fingering, the fluxes, averaged over intervals of 30 to 60 min, have been parameterized in terms of Δs and $\alpha\Delta T/\beta\Delta s$ across the interface. For the diffusive regime, ΔT and $\beta\Delta s/\alpha\Delta T$ are the key parameters (α is the thermal expansion coefficient of seawater, and β the coefficient of haline contraction). We have used the Marmorino and Caldwell flux relationship¹³ for the diffusive regime, and the expression given by Schmitt¹⁴ for fingering interfaces. Measurements of the thickness of interfaces in the diffusive regime⁴ and the diameter of salt fingers⁸ have given confidence that the laboratory observations scale satisfactorily to oceanic situations.

Laboratory salt fingering experiments have demonstrated that: (1) a steady shear flow changes the structure of the double diffusive instability from fingers to sheets, but does not alter J_b (ref. 15); (2) externally generated turbulence inhibits the J_b produced by fingering, the more so with increasing turbulence levels^{16,17}. Therefore, the flux relationships will give overestimates where there is externally generated turbulence as well as salt fingering.

Profiles in the Bahamas

Three laterally coherent patches with enhanced ϵ levels were found in the set of profiles from the Bahamas (Fig. 1). The most prominent patch occurs below the Ts maximum near 1 MPa, where the Ts relation is diffusively unstable to salt fingering.

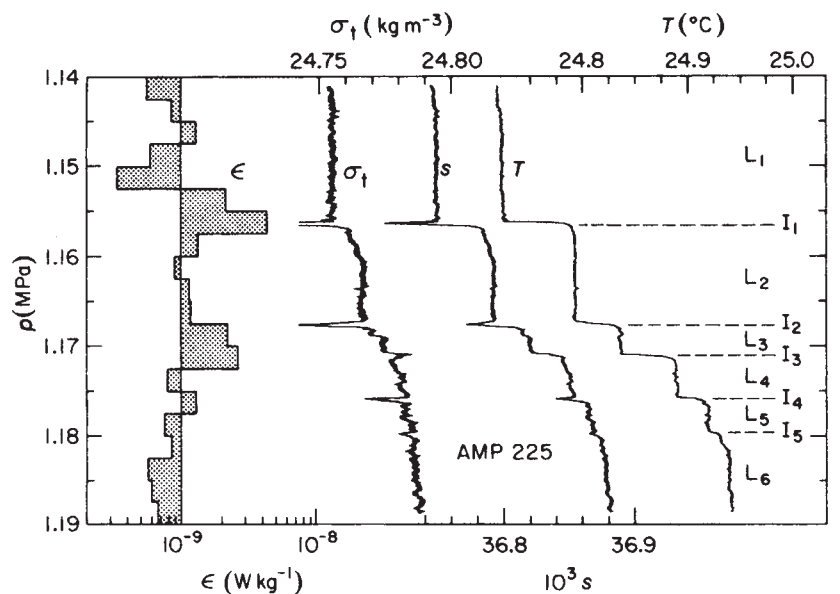


Fig. 2 Detailed plot of the diffusive steps in AMP 225 from the Bahamas data. Mismatches in the dynamic responses of the temperature and conductivity sensors are responsible for the sharp spikes and the apparent stratification within the thinner layers. For the interfaces, $\beta\Delta s/\alpha\Delta T = 1.57, 1.54, 1.52, 1.36$, and 1.17 , from top to bottom. For layers L_1 to L_6 , $10^9 \epsilon = 1.2, 1.1, 2.2, 0.8, 0.8$, and 0.6 . For interfaces I_1 to I_5 , $10^9 J_b = 4.0, 2.3, 2.8, 1.3$, and 0.5 .

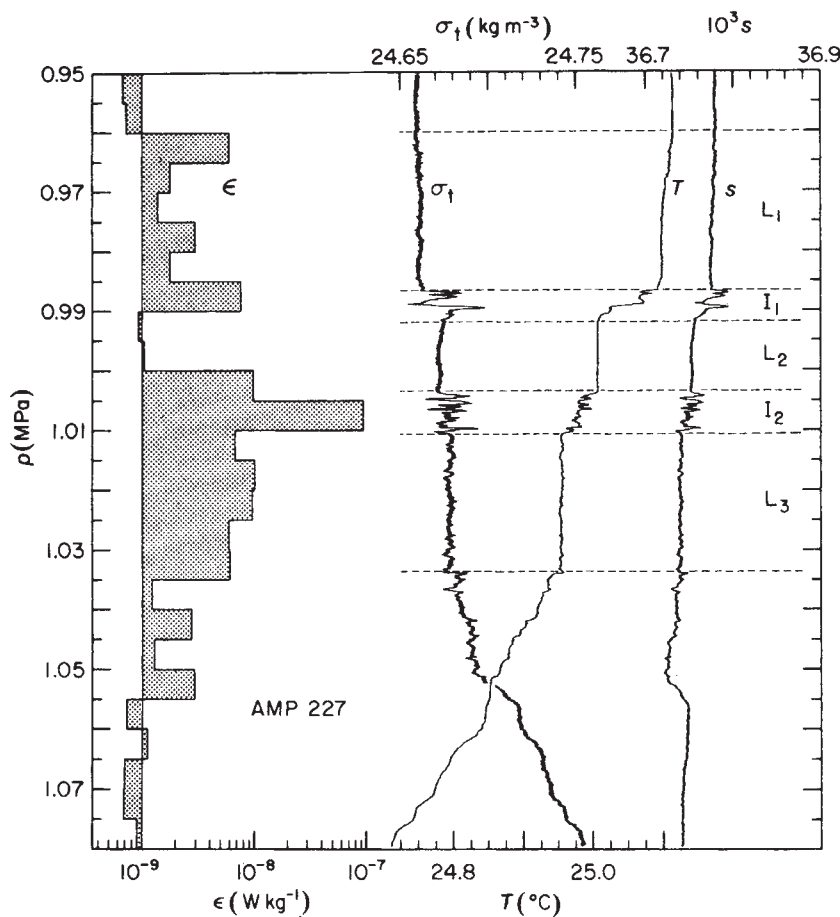


Fig. 3 Expanded scale plot of a high ϵ patch in a region unstable to salt fingering. The section is from AMP 227, which was taken in the Bahamas. There is a net density increase across both interfaces between the three layers in the centre of the patch; the spikes in salinity and density are instrumental artefacts. $\alpha\Delta T/\beta\Delta s = 1.73$ and 1.05 for the upper and lower interfaces, respectively. For layers L_1 through L_3 , $10^9 \epsilon = 2.8, 1.4$, and 7.7 . For interfaces I_1 and I_2 , $10^9 \epsilon$ and $10^9 J_b = 11.0, 94.0$, and $1.7, 0.9$, respectively.

Averaged across the five profiles, $\bar{\epsilon} = 7 \times 10^{-9} \text{ W kg}^{-1}$. The second patch occurs above the Ts maximum near 1.2 MPa , where families of diffusive steps are found in all profiles. This patch is weaker, with $\bar{\epsilon} = 1.7 \times 10^{-9} \text{ W kg}^{-1}$. The third patch occurs only in the three profiles to the left, and is above the uppermost Ts maximum; $\bar{\epsilon} = 4.7 \times 10^{-9} \text{ W kg}^{-1}$. In individual patches the average dissipation differed from $\bar{\epsilon}$ by less than $\pm 50\%$, which is justification for neglecting the time derivative in equation (1). (The ϵ estimates are obtained by direct integration of spectra of small-scale velocity, assuming spectral isotropy in the dissipation range. Because the spectra were integrated over a fixed wavenumber band, $2\text{--}50$ cycles per metre, ϵ values near 10^{-9} contain some noise contribution and are overestimates.)

A more detailed plot of the diffusive steps in the middle profile is given in Fig. 2. Five distinct layers extend downward from the local Ts minimum. The greater thickness of the upper layers suggests that the diffusive layering developed at the Ts minimum, and that the initial layers merged as the diffusive structures grew downward¹². Comparison of the measured ϵ values with the values for J_b computed from ΔT and Δs reveals that the ratio ϵ/J_b varies from $1/2$ to $1/6$, compared to an expected value of 1 . For example, for L_2 , $\epsilon = 1.1 \times 10^{-9}$, compared with a net J_b for I_1 and I_2 of 6.3×10^{-9} .

To produce $\epsilon/J_b < 1$, either the measured $\bar{\epsilon}$ values are lower than the true values because of statistical intermittence or the estimated J_b values are too high because of inaccuracy or inap-

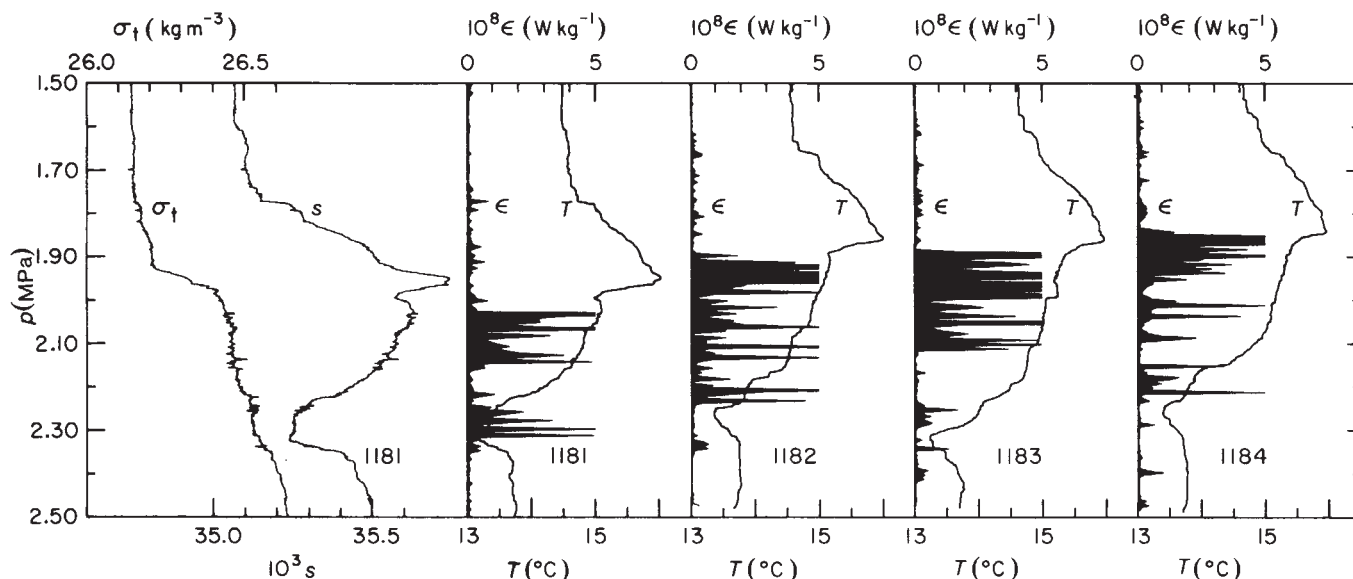


Fig. 4 A sequence of four profiles taken during 1 h within a warm-core ring. The average spacing was 220 m. The format is similar to that in Fig. 1 except that ϵ is plotted on a linear abscissa and truncated at $5 \times 10^{-7} \text{ W kg}^{-1}$; maximum values are $2 \times 10^{-7} \text{ W kg}^{-1}$. The 35-m-thick zones beneath the T_s maxima contain ϵ values 10 times greater than any found elsewhere in these profiles. In this patch, $\bar{\epsilon} = (1.7\text{--}2.6) \times 10^{-8} \text{ W kg}^{-1}$. A much weaker increase in ϵ occurs in the high gradient region above the T_s maximum; $\bar{\epsilon} = (1.3\text{--}1.9) \times 10^{-9} \text{ W kg}^{-1}$. Above this region, $\bar{\epsilon} = 5 \times 10^{-10} \text{ W kg}^{-1}$, which is the noise level of these ϵ measurements.

plicability of the laboratory flux relationships. The former case seems improbable because of the small variability in the $\bar{\epsilon}$ values observed across the five profiles and the bias towards high values due to the noise contribution. The most likely situation is that no turbulent production by the Reynolds stress occurred and the J_b estimates are too high. If the other flux relationship¹⁸ is used, ϵ/J_b is decreased by about 50%. Because both flux relationships are based on only a few data points in the $\beta\Delta s/\alpha\Delta T$ range of these interfaces, errors in the relationships appear to be likely.

The patch of high ϵ in AMP 227 is 10 m thick and is terminated by well-stratified regions above and below (Fig. 3). The patch is centred on three well-mixed layers. Within the upper and lower layers, $\epsilon/J_b = 1.6$ and 8.6, respectively. The dissipation rate throughout most of the middle layer is much lower; most of the contribution to ϵ comes from activity at the base of the layer. For this layer, $\epsilon/J_b = 0.5$. In this patch $\bar{\epsilon}$ varies no more than $\pm 50\%$ in the five profiles; thus, the $\bar{\epsilon}$ value used above can be regarded as an ensemble average. Because the ΔT and Δs steps in AMP 227 are the largest in the group of profiles, the ϵ/J_b ratios are minimum estimates. If $\epsilon/J_b > 1$, two explanations are possible: the laboratory flux relationships underestimate the oceanic case, or part of the turbulent dissipation is produced by the Reynolds stress.

If salt fingering was occurring, it would have been across the two interfaces. Interface I_1 , with a strong gradient in the centre and a transition region on either side, resembles the internal structure of fingering interfaces in laboratory observations¹⁹. Inversions like those in the upper transition region have also been observed in the laboratory, where they have been identified as intermittent instabilities produced by the destabilizing buoyancy flux¹⁹. This may account for the relatively high dissipation rate within the interface; $\epsilon/J_b = 6.5$. However, if fingering was occurring, J_b should have been the same in the well-mixed layers immediately above and below the interface. The much lower ϵ levels immediately below the interface do not seem consistent with fingering. The temperature profile within the lower interface, I_2 , has small-scale inversions throughout, with no monotonic section where vertically-aligned fingers might be expected. This indicates either tilted fingers, due to shear across the interface¹⁵, or overturning, due to a shear instability. The very high dissipation, $\epsilon/J_b = 95$, suggests that Reynolds stress production is at least partly responsible.

In summary, increases in ϵ have been observed in those

sections of the Bahamas profiles where the T_s relation is diffusively unstable, with the largest increases occurring where salt fingering is possible. In sections containing diffusive steps, ϵ/J_b was lower than expected by at least 1/2 to 1/6. This implies that the laboratory flux laws are giving overestimates of J_b . In sections where salt fingering is possible, some of the ϵ/J_b values are greater than one, in some cases by large amounts. This can be due to underestimation of J_b by the laboratory flux laws or to Reynolds stress production. The step structures, the structure of I_1 , and the average ϵ/J_b ratios for L_1 and L_2 are all consistent with fingering across the interfaces. However, the relatively high ϵ/J_b in L_3 and the very high ϵ/J_b and the temperature inversions in I_2 do not agree with an interpretation of simple salt fingering, but suggest at least some external turbulent production.

Profiles in a warm-core Gulf Stream ring

All of the profiles collected during a 2-week period within the warm-core ring showed a salt-stabilized temperature inversion between the base of the mixed layer and a T_s maximum near 2 MPa. This feature extended across the ring, and appeared to have been caused by the 'flooding' of the ring by cooler, less saline slope water (T. Jpyce, personal communication). A prominent patch of high ϵ begins slightly below the T_s maximum and extends 35 m to the T_s minimum (Fig. 4). Averaged across the four profiles, $\bar{\epsilon} = 2.2 \times 10^{-8} \text{ W kg}^{-1}$; individual averages vary less than $\pm 25\%$. A weak increase in ϵ occurs in the high-gradient region above the T_s maximum; $\bar{\epsilon} = 1.6 \times 10^{-9} \text{ W kg}^{-1}$. Only a few diffusive steps occur in this region.

The high ϵ patch in AMP 1183 contains the largest ΔT and Δs steps in the four profiles (Fig. 5). The interface in the middle separates two weakly stratified layers of high ϵ and intense thermal microstructure. No other interfaces that could support salt fingers are evident. For the upper and lower layers, $\epsilon/J_b = 14$ and 5.1, respectively. These ratios are somewhat higher than those in the Bahamas profiles and, as in the Bahamas data, are the minimum ratios that can be formed from the set of profiles. To evaluate the possibility of turbulent production by the Reynolds stress, two velocity profiles are available.

In both XCP profiles the maxima and minima of temperature and velocity occur at the same pressures (Fig. 6). The correspondence between the temperature maximum and the V component of velocity in the upper panel is particularly striking, and corresponds with the density step. Consequently, large average shears occur over the thick weakly stratified regions above and below

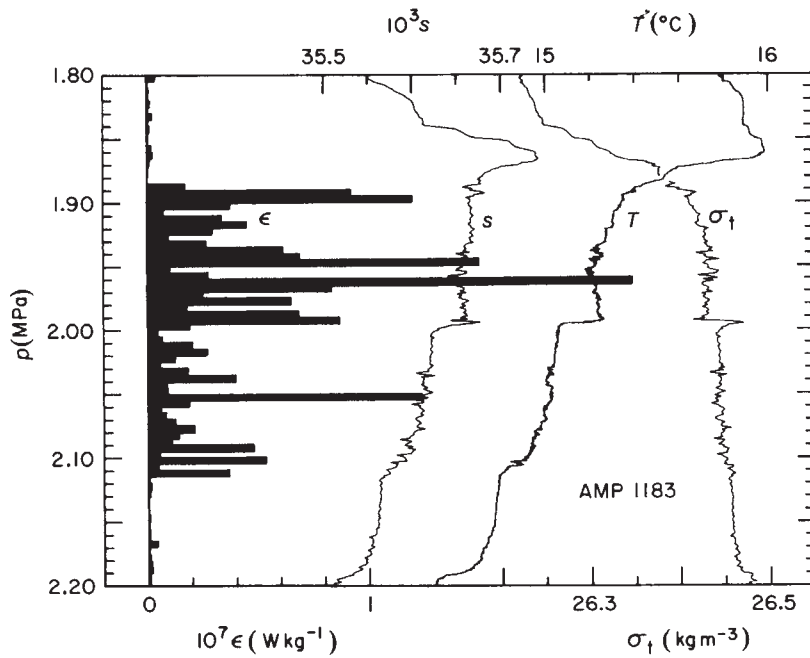


Fig. 5 Expanded scale plot of a high ϵ patch in a profile from the warm-core ring. The high ϵ levels terminate at the density gradients above and below the 24-m-thick patch. If salt fingering was occurring, it should have been across the sharp interface in the centre. $\alpha\Delta T/\beta\Delta s = 1.68$ for the interface, and $J_b = 5.3 \times 10^{-9} \text{ W kg}^{-1}$. Above and below the interface, $\bar{\epsilon} = 5.3 \times 10^{-8} \text{ W kg}^{-1}$ and $2.2 \times 10^{-8} \text{ W kg}^{-1}$, respectively. Unlike the example in Fig. 4, the highest ϵ levels are found within the layers and not within the interface.

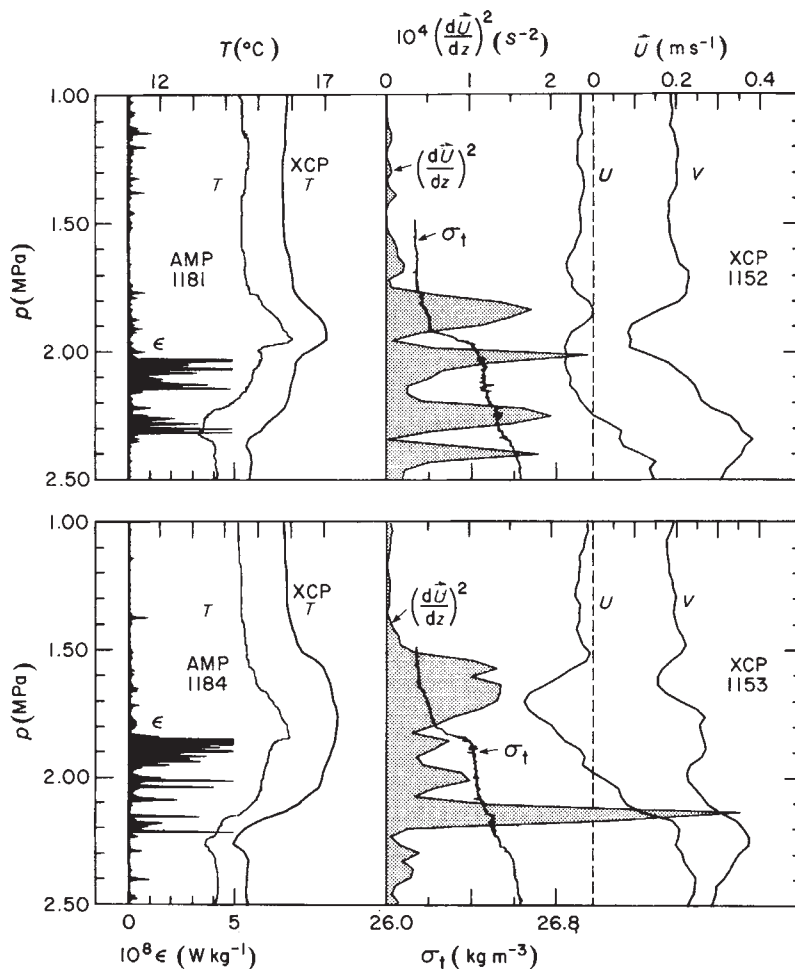


Fig. 6 Comparison of XCP profiles taken before and after the series of AMP drops in the warm-core ring. XCP 1152 was taken 1 h before and 1 km removed from the location of AMP 1181. XCP 1153 was taken 1 h after and 1/2 km from the site of AMP 1184. The panels on the left display the 1/2 m averages of ϵ and T from AMP, and T from XCP. The pressure scales of the XCPs were adjusted to produce the best agreement of the AMP and XCP temperature records; the XCP pressure is based on a mean fall rate, not on measured pressure. The panels on the right display, from left to right: $|\partial \bar{U}/\partial x_3|^2$, σ_t from AMP, the east component of velocity (U), and the north component of velocity (V). The velocities are not absolute, but are relative to the bottom of the XCP record, at 800 m. Note that the local maxima in temperature and velocity occur at the same depths, and that $|\partial \bar{U}/\partial x_3|^2$ has large increases above and below the T_s maximum.

the T_s maximum. Above the maximum, $|\partial \bar{U}/\partial x_3| = 0.011 \text{ s}^{-1}$ in both profiles. Below the maximum, the mean shear is 0.011 s^{-1} in XCP 1152 and 0.008 s^{-1} in XCP 1153. A local minimum in shear occurs across the density step at the nose of the near-inertial feature.

A necessary condition for dynamic instability is given in terms of the Richardson number:

$$Ri \equiv \frac{N^2}{|\partial \bar{U}/\partial x_3|^2} < \frac{1}{4} \quad (3)$$

where N is the stability frequency.

Using gradients averaged over both sets of AMP and XCP profiles gives: $Ri = 0.26 \pm 0.11$ above the T_s maximum; $Ri = 9.6 \pm 3.4$ across the density step at the T_s maximum; and $Ri = 0.20 \pm 0.16$ across the 35-m-thick high ϵ region below the T_s maximum. Because the shear and density measurements were made with different instruments and were not simultaneous, instability below the T_s maximum cannot be demonstrated conclusively. However, the coincidence of the low Ri region with the high ϵ patch is certainly suggestive of turbulent produc-

tion by the Reynolds stress. The reduction of the salt fingering buoyancy flux by externally generated turbulence in laboratory experiments adds further weight to the conclusion that the Reynolds stress was important in this patch.

Conclusions

Laterally-coherent zones of enhanced ε have been found above and below T_s maxima in the thermohaline intrusions, where the T_s relation is diffusively unstable. Distinctive steps in temperature and salinity above some of the T_s maxima are signatures of the diffusive regime of double diffusion. In the one case examined in detail, the ε/J_b ratios were no more than 1/2 to 1/6 of the values expected from laboratory flux relationships. Thus, there is no evidence for turbulent production by the Reynolds stress. The most probable cause of the discrepancy is the inadequate coverage of the low $\beta\Delta s/\alpha\Delta T$ range by the laboratory experiments.

Where the T_s relation was diffusively unstable to salt fingers, $\bar{\varepsilon}$ was a factor of 5 to 10 higher than where the T_s relation was unstable to the diffusive regime. These patches were centred on one or two sharp interfaces, which are possible sites for salt fingering. In one of the profiles from the Bahamas, a series of three well-mixed layers was found, which had $\bar{\varepsilon}/J_b = 1.6, 0.5$, and 8.6. The case investigated in detail from the warm-core ring had $\bar{\varepsilon}/J_b = 14$ and 5. Other patches did not contain distinct layers, but were weakly stratified mixing zones. The high ε/J_b ratios could be due to an underestimate of J_b by the use of laboratory flux relationships or to the production of additional turbulence by the Reynolds stress. The character of the profiles and the high shear levels, in the case of the ring, make it likely that Reynolds stress production is at least partly responsible.

In considering mechanisms by which the intrusions in the warm-core ring could have been formed, it is necessary to consider the nature of the velocity field. The velocity structures across the T_s maximum are similar to those of near-inertial internal waves²⁰. Both profiles have a clockwise rotation of the velocity vector with increasing pressure, consistent with downward propagation. Also, when the profiles are rotated inertially to a common origin in time, they appear to be the same structure, as do several other profiles taken within 10 km and 18 h. Therefore, we have considered three mechanisms by which inertial motions could be causally related to the intrusion.

The first mechanism is the formation of the intrusion by lateral, double diffusive instability across the T_s front at the ring edge. In this case, the near-inertial feature would be the remnant of the internal waves generated by the initial spreading of the intrusion. Support for this interpretation comes from the similarity of the observed profiles to ones observed in laboratory intrusions formed by double diffusion. The relationship between the density field, the mixing patches, and the velocity field in Fig. 6 is similar to that observed through double diffusive intrusions in the laboratory by Thorpe *et al.*²¹. In particular, comparison of the density field (their Fig. 12) with photographs of dye streaks (their Fig. 11) shows vigorous mixing in the lower, weakly stratified part of the intrusion and laminar flow in the strongly stratified transition between different intrusions. If the intrusion was formed by lateral, double diffusive instability, it would be expected to slope across density surfaces²². However, when we plotted the temperature and salinity profiles against density as the vertical coordinate, we found no slope greater than 1.4 m vertically over 1 km horizontally. This indicates that, although double diffusion locally altered the density profile, it was not the dominant factor in forming the intrusion.

A second formation mechanism is lateral displacements across the strong T_s front at the ring edge by the near-inertial motions. This mechanism was considered previously by Georgi²³ in inter-

preting a data set, but rejected; Georgi concluded that the near-inertial energy in the Garrett-Munk spectrum²⁴ was insufficient to produce intrusions in the Antarctic Circumpolar Front. Use of the Garrett-Munk spectrum can be objected to on the grounds that it is a representation of temporally and spatially averaged internal wave energy levels and is not indicative of the instantaneous levels in energetic locations. The spectrum also underestimates the energy in the near-inertial band, even at background open-ocean sites. Recently, an enhancement of near-inertial energy in frontal regions, compared with the background open-ocean levels, has been discovered. The level of near-inertial energy found in the North Pacific Subtropical Front was four times greater than background²⁵, and that in 'our' warm-core ring was a factor of seven higher (E. Kunze, personal communication). Clearly, the formation of intrusions in frontal regions by near-inertial motions should be re-examined. In the present case, the observed near-inertial particle motions do not have the energy to reach the front at the edge of the ring. The displacement radius is given by $r = |\bar{U}|/\omega_i$, where ω_i is the local inertial frequency in radian units. For the speed of this feature, 0.2 m s⁻¹, the inertial radius is only 2.2 km, compared with a distance of 20 km to the edge of the ring. The persistence of the T_s maximum is also inconsistent with formation by near-inertial motions; in such a case the T_s feature should have an inertial periodicity.

The third mechanism is generation of the near-inertial motions by the formation of the intrusion when the ring was 'flooded'. This probably did not happen uniformly across the ring, which accounts for the presence of near-inertial motions at the T_s maximum is only half of all XCP profiles. The structure of density profiles and mixing patches suggests the subsequent local modification of the profile by double diffusion as well as by shear-generated turbulence. The interaction of shear and double diffusion in a rotating system has recently been demonstrated by stability analysis³⁶.

None of the formation mechanisms can be demonstrated conclusively with the limited data used in this preliminary analysis. However, the observed coincidence of the T_s maxima and minima with the local velocity maxima suggests a link between the scalar and velocity fields that should be present in other oceanographic settings. Observations similar to ours are needed in these settings to resolve which formation mechanisms are operative. Among the places for future work are the prominent salt fingering layers found beneath the Mediterranean outflow and near Barbados. For example, J_b values estimated for the salt fingering steps near Barbados²⁷ are $(1.0-2.6) \times 10^{-8}$ W kg⁻¹. Because those salt fingers are not connected with thin intrusions, the relative importance of the turbulent production terms should be different.

To make maximum use of ε/J_b in examining double diffusive mixing in the ocean, additional laboratory experiments are needed. Measurements of ε and J_b should be taken in both regimes of double diffusion. In particular, data are needed for $\beta\Delta s/\alpha\Delta T$ and $\alpha\Delta T/\beta\Delta s$ between 1 and 2, which is the range of most of the strong oceanic signatures of double diffusion. Because oceanic observations are either single realizations or averages over short intervals, these measurements should determine both the time-averaged ε and J_b values and their intermittence.

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Nucleotide sequences of cloned cDNAs for two types of bovine brain substance P precursor

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The primary structures of two types of bovine brain substance P precursors have been determined. One precursor contains a sequence homologous to that of the amphibian peptide kassinin. This new tachykinin sequence is bounded by paired basic amino acids Lys-Arg, which suggests that, like substance P, it can be liberated from the precursor and may serve as an endogenous hormone or neuromediator in mammalian organisms.

SUBSTANCE P was first isolated from brain and intestine more than 50 years ago¹. Although many of the peripheral actions of substance P, such as smooth muscle contraction and hypotension, had been established, it was not until 1971 that Leeman and her colleagues purified the peptide and determined its structure as an undecapeptide Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂ (ref. 2). Evidence has been accumulating that substance P may act as a neurotransmitter in primary sensory neurones. Substance P immunoreactivity has been shown to be present mainly in small unmyelinated fibres and cell bodies as well as in nerve terminals of many peripheral ganglia (reviewed in ref. 3), and it has been shown that substance P is released on nerve depolarization and can alter the activity of some neurones when applied in their vicinity⁴. Substance P therefore provides an attractive system for studying the mechanism of peptidergic neurotransmission or neuromodulation among many other small peptides now known to exist in the central nervous system (CNS).

Several structurally related peptides, termed tachykinins, with biological activities similar to substance P, have been isolated from non-mammalian tissues; these peptides include physalaemin, kassinin, uverolein and eldoisin⁵. Peptides in the tachykinin family possess a common C-terminal sequence Phe-X-Gly-Leu-Met-NH₂ which accounts for the fundamental properties of the tachykinins, and the specific action of each member of the group depends on its unique N-terminal sequence. In mammalian species, substance P has been regarded as the only peptide representing the tachykinin family. However, the recent finding of physalaemin-like immunoreactivity in mammalian tissues has raised the possibility that tachykinins other than substance P may exist in mammalian species⁶. Moreover, it has been found that non-mammalian kassinin and eldoisin are considerably more potent than substance P itself in some peripheral actions as well as in some central nervous effects in mammals^{5,7}. The question of whether tachykinins other than substance P are present in mammalian tissues is crucial in understanding the physiological role of the tachykinin system in mammals.

It is generally accepted that a small biologically active peptide such as substance P is initially synthesized as a larger precursor. Little is known, however, about the origin of substance P, and the relationship between substance P and other tachykinins is poorly understood. The construction of bacterial plasmids containing the mRNA sequence encoding the substance P precursor is a direct approach to analysing the amino acid sequence, the

mRNA and the gene for the precursor. Furthermore, such an approach might also lead to the characterization of hitherto unknown tachykinins within the same precursor, in the way that several novel peptides such as γ -melanotropin (γ -MSH), calcitonin gene-related peptide (CGRP) and multiple egg-laying peptides were found within the ACTH- β -EPH precursor (pre-proopiomelanocortin), the calcitonin precursor and the egg-laying hormone precursor respectively⁸⁻¹⁰.

We report here cloning of DNA sequences complementary to the bovine striatal mRNAs coding for the substance P precursors. Nucleotide sequence analysis of cloned cDNAs has revealed that the bovine brain substance P precursors are encoded by at least two different mRNAs. One encodes not only the substance P sequence but also a kassinin-like sequence, while the other lacks the latter sequence, thus encoding a single substance P sequence. Because of these characteristic structures, we refer hereafter to these precursors as preprotachykinins.

Isolation of cDNA clones

Experimental details of cloning cDNAs for preprotachykinins are described in Fig. 1 legend. In brief, our initial approach for the isolation of cloned cDNA sequences for preprotachykinins involved the chemical synthesis of two mixtures of tetradecamer oligodeoxyribonucleotides representing all possible complementary sequences corresponding to the middle portion (oligo I) and the C-terminal portion (oligo II) of the amino acid sequence of substance P respectively. The oligo II mixture was first used to enrich cDNA for preprotachykinin by specific reverse transcription of bovine striatal poly(A) RNA. The cDNA transcripts thus formed were cloned and screened by hybridization with the oligo I probe, the sequence of which is located upstream from the sequence represented by the oligo II primer. After isolating cDNA clones carrying a partial preprotachykinin mRNA sequence, an appropriate restriction fragment derived from the isolated cDNA clone, in turn, was used for screening a cDNA bank representing bovine striatal poly(A) RNA sequences. In the first screening, two hybridization-positive clones (clones pSP2 and pSP3) were isolated and both clones were found to contain a cDNA sequence encoding substance P (Fig. 1). In the second screening, four hybridization-positive clones were isolated and classified by restriction endonuclease mapping into two groups, each including two clones. The larger cDNA inserts in each of clones (pSP306 and pSP307) were subjected to nucleotide sequence analysis according to the strategy indicated in Fig. 1 legend. In addition, the nucleotide sequence

differences in the protein-coding regions between the two types of cDNA insert (see below) were confirmed by a further sequence analysis of the cDNA inserts in the two remaining clones pSP301 and pSP302 (Fig. 1).

Preprotachykinins and their mRNAs

Figure 2 shows the primary structures of bovine preprotachykinin mRNAs deduced from the two types of cDNA sequence. A comparison of the nucleotide sequence of the 1,082-base pair (bp) insert (residues -143 to 939) of clone pSP307 and that of the 1,002-bp insert (-117 to 885) of clone pSP306 exhibits 54 consecutive nucleotide deletions/additions and two nucleotide substitutions in their overlapping regions. In both mRNAs, the sequence of nucleotide residues 172-204 corresponds precisely to the amino acid sequence of substance P. As predicted (see Fig. 1 legend), the C terminus of substance P is followed by the glycine residue, which could thus serve as a donor of the amide group for the C-terminal methionine residue of substance P. The glycine residue is followed by the dibasic amino acid residues Lys-Arg, which apparently represent the site of proteolytic processing. Interestingly, the N-terminal processing point of substance P is located between the sequence Arg-Arg rather than after the dibasic structure.

The translational initiation site is assigned to the methionine codon AUG at position 1-3 because this is the first AUG triplet that appears downstream from the nonsense codon UGA (residues -102 to -100) found in frame. The N-terminal sequence starting with this methionine exhibits a feature characteristic of the signal peptide present in other secretory proteins¹¹; 12 out of 13 amino acid residues in the N-terminal sequence consist of hydrophobic amino acids. Such signal peptides are usually 15-30 amino acids long, contain a large number of hydrophobic amino acids and terminate in an amino acid with a small neutral side chain¹² (for example, alanine, glycine or serine). Therefore, a possible site for cleavage of the signal peptide of preprotachykinins would be after position 18 (Ser), 19 (Ala), 23 (Gly) or 24 (Ala).

The deduced amino acid sequence in the cryptic portion of one of the preprotachykinins (named β -preprotachykinin) indicates that the sequence of amino acids 98-107 is strikingly similar to the sequence of an amphibian tachykinin, kassinin. This segment of the precursor protein contains the common tachykinin sequence Phe-X-Gly-Leu-Met and shares lysine, aspartic acid and valine residues with kassinin at equivalent positions (Fig. 3). Moreover, the C-terminal methionine residue is followed by the glycine residue and the peptide fragment that has been discovered is flanked on both sides by a pair of basic amino acid residues. This suggests that the new peptide, like substance P, can be liberated from the precursor by proteolytic processing, and that the methionine amide can be formed at the C terminus of this peptide during processing. In view of the remarkable structural homology of the newly found peptide fragment with kassinin and the possible existence of this peptide in mammalian tissues as discussed below, we have named the peptide substance K. The N-terminal residue of substance K, like substance P, could be arginine at position 97, but it seems more likely that the N-terminal cleavage site is immediately after the paired basic amino acids Lys-Arg, because a survey of various prohormones suggests that the Lys-Arg pair flanking the peptide sequence is generally removed during processing¹².

The second type of preprotachykinin (named α -preprotachykinin) exhibits an interesting structural relationship with β -preprotachykinin. The former sequence shows 18 consecutive amino acid deletions and one amino acid replacement as compared with the latter sequence, but the two sequences are otherwise identical. Because the deleted amino acid sequence includes the whole substance K sequence, α -preprotachykinin contains a single substance P sequence.

The termination codon UAG follows four consecutive basic amino acid residues Arg-Arg-Arg-Lys. α - And β -preprotachykinins are composed of 112 and 130 amino acid residues respectively, having calculated molecular weights of 13,109 and 15,076

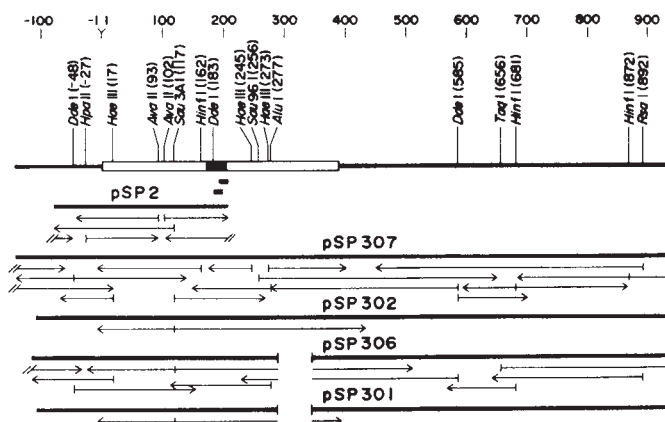
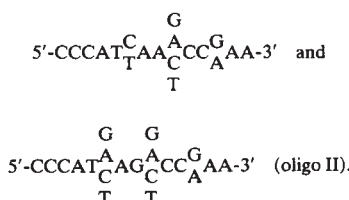


Fig. 1 Strategy of sequencing the cDNA inserts in clones pSP2, pSP301, pSP302, pSP306 and pSP307. The restriction map displays only relevant restriction endonuclease sites, which are identified by numbers indicating the 5'-terminal nucleotide generated by cleavage; for the nucleotide numbers, see Fig. 2. □, Sequence corresponding to coding region ■, coding region for substance P. The sequences of synthetic oligodeoxyribonucleotides used for specific priming of reverse transcription and for probing cloned cDNAs for preprotachykinin are indicated by small solid boxes directly beneath the restriction map. Gaps on the bars of clones pSP301 and pSP306 represent 54-nucleotide deletions. The locations of the 5' terminus of pSP301 and pSP302 are approximate. The direction and extent of sequence determinations are shown by horizontal arrows under each clone used; the sites of 5'-end-labelling are indicated by short vertical lines at the ends of arrows. The slash marks at the ends of arrows mean that the site of 5'-end-labelling was located on the vector DNA.

Methods: Two mixtures of tetradecamer oligodeoxyribonucleotides were synthesized by the modified triester methods²⁴; one is composed of

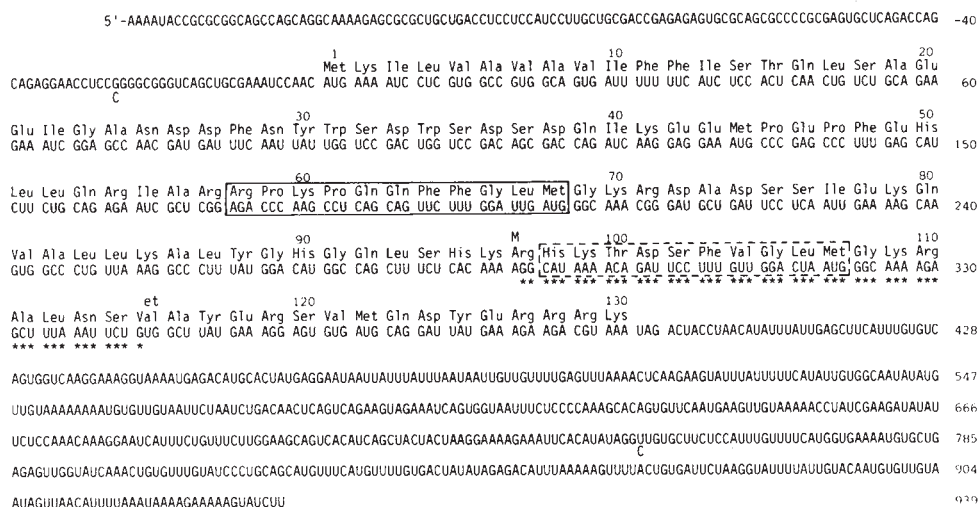


and the other of



The oligo I and oligo II mixtures represent all possible complementary sequences corresponding to the fifth to ninth amino acid residues of substance P, Gln-Gln-Phe-Phe-Gly, and those corresponding to the C-terminal tetrapeptide sequence of substance P followed by the glycine residue Phe-Gly-Leu-Met-Gly respectively (excluding the third nucleotide residue of the C-terminal glycine codon in both sequences); in the latter pentapeptide sequence, the amide group of the C-terminal methionine of substance P was predicted to be derived from the amino group of the glycine residue following the methionine residue, because such an amidation mechanism was reported for many other peptide hormones terminating in an α -amide group (ref. 25 and refs therein). Clone pSP2 was isolated as follows. Total RNA was extracted²⁶ from bovine striata, because it was reported that a high level of substance P biosynthesis occurs in the striatum²⁷ (caudate nucleus plus putamen). Poly(A) RNA was obtained by hybridization³¹ at 36 °C with the 5'-³²P-labelled oligo I synthetic oligodeoxyribonucleotide probe, and two hybridization-positive clones (pSP2 and pSP3) were isolated (data for clone pSP3 are not shown). For the isolation of clones pSP301, pSP302, pSP306 and pSP307, a cDNA library was constructed by the method of Okayama and Berg³² using 4 μ g of bovine striatal poly(A) RNA and 3 μ g of the vector-primer DNA. Approximately 200,000 ampicillin-resistant transformants were screened³³ by hybridization at 60 °C with the Dde I fragment (residues -48 to 182 in Fig. 2) derived from the clone pSP2; the Dde I fragment was labelled by nick-translation²³ with [α -³²P]dCTP. DNA sequencing was carried out by the procedure of Maxam and Gilbert³⁴.

Fig. 2 Primary structures of two preprotachykinin mRNAs. The nucleotide sequences of α - and β -preprotachykinin mRNAs were deduced from those of the cDNA inserts in clones pSP306 and pSP301, and clones pSP307 and pSP302, respectively. According to the nucleotide sequence of β -preprotachykinin mRNA, nucleotide residues are numbered in the 5' to 3' direction, beginning with the first residue of the AUG triplet encoding the initiative methionine, and the nucleotides on the 5' side of residue 1 are indicated by negative numbers. Only the nucleotide differences of α -preprotachykinin mRNA when compared with β -preprotachykinin mRNA are displayed below the sequence of β -preprotachykinin mRNA; an asterisk indicates a deletion of the corresponding nucleotide residue -117. The predicted amino residues are numbered beginning with dashed lines respectively; the C-terminally defined. The replacement preprotachykinin. Those amino acid



respectively. By contrast with most other eukaryotic mRNAs¹³, a preference for A or U in the third position on the codons is observed in preprotachykinin mRNAs. The 3'-untranslated region of the mRNAs is 549 nucleotides long [excluding the poly(A) tail]. The sequence AAUAAA commonly found near the 3' end of eukaryotic mRNAs¹⁴ is present in preprotachykinin mRNAs 15 nucleotide residues upstream from the poly(A) tail.

Because we have identified four individual clones, two of which contain the cDNA sequence specific for either α - or β -preprotachykinin, we have concluded that bovine preprotachykinins are encoded by at least two different mRNAs. To confirm this conclusion, we also performed the following S_1 nuclease mapping experiments¹⁵ using two kinds of cDNA probe. The nick-translated 402-nucleotide *DdeI* fragment (residues 183–584) derived from the cDNA insert of clone pSP307 was hybridized to total striatal poly(A) RNA. Hybridization of this cDNA probe to α -preprotachykinin mRNA should result in the formation of a single-stranded cDNA loop corresponding to the deleted portion of the mRNA, so we expected that S_1 nuclease would split the cDNA probe into two DNA fragments of 244 and 104 nucleotides (Fig. 4c). In fact, DNA bands with mobilities corresponding to sizes of approximately 240 and 100 nucleotides were observed in the autoradiograph of the products of S_1 nuclease digestion (Fig. 4a). An additional band of approximately 400 nucleotides could be accounted for by the duplex formation between the cDNA probe and β -preprotachykinin mRNA, or by the self-annealing of the cDNA probe, or by both. Thus, another S_1 nuclease mapping experiment was carried out by use of the 5'-end-labelled 1,089-nucleotide *TaqI* fragment derived from clone pSP307. This DNA fragment contained the nucleotide sequence complementary to 800 nucleotides of the 5' sequence of β -preprotachykinin mRNA, 10 nucleotides of the oligo(dC) tail and also 279 nucleotides of the vector sequence. Two bands with mobilities corresponding to sizes of approximately 800 and 310 nucleotides appeared after S_1 nuclease digestion (Fig. 4b). The former accorded with the size extending from the *TaqI* site to the 3' end of the antimessage strand of the cDNA probe, while the latter corresponded to the length ending at the deleted portion of α -preprotachykinin mRNA (Fig. 4d). These S_1 mapping experiments therefore demonstrate that α - and β -preprotachykinin mRNAs are indeed present in the bovine striatum.

Structural implications

The structures of two bovine preprotachykinins are schematically illustrated in Fig. 5. β -Preprotachykinin contains two

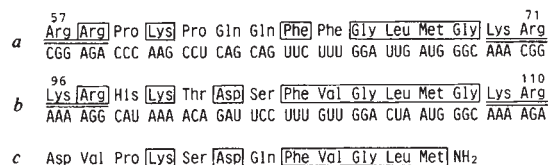


Fig. 3 Comparison of nucleotide and amino acid sequences in the regions of substance P (*a*) and substance K (*b*) and amino acid sequence of amphibian kassinin⁵ (*c*). For amino acid numbers, see Fig. 2. Amino acid residues identical in the regions of substance P and substance K or in the sequences of substance K and kassinin are enclosed by solid lines. Two consecutive basic amino acids are marked by double underlining. The methionine residue of substance P at position 68 is in the form of an amide.

tachykinin sequences, while α -preprotachykinin includes a single substance P sequence. In both preprotachykinins, every unit composed of a tachykinin sequence is bounded by paired basic amino acids lysine and arginine, although the substance P sequence includes the arginine residue corresponding to the second amino acid residue of the dibasic structure located at the N-terminal flanking region. The finding of the novel structure, substance K, within the precursor molecule suggests that this peptide can be liberated from the precursor by proteolytic processing. In this context, it is interesting that substance K has a chemical property similar to the substance P-like peptide reported by Keen *et al.*¹⁶. They have observed that rat dorsal root ganglia incubated with ³⁵S-methionine can synthesize authentic substance P and also a peptide that is immunoprecipitated by C-terminal- but not N-terminal-specific anti-substance P antisera. Moreover, analysis of ³H-phenylalanine or ³H-proline incorporation revealed that the novel peptide contains only one phenylalanine and no proline residue. Thus, there is apparently a coincidence in the chemical properties of the novel peptide and substance K, suggesting that the former may represent substance K liberated from β -preprotachykinin.

If substance K indeed exists in mammalian tissues, what physiological significance can be attributed to this peptide? Erspamer was the first to point out that members of the tachykinin family exhibit considerable differences in their relative potencies in different tests, although they share a common spectrum of biological activities⁵. Iversen then extended this analysis and consequently introduced the terminology SP-P and SP-E receptors to represent two extreme types of biological activity⁷. The SP-P receptor exhibits greatest selectivity for substance P and physalaemin, predominates in the guinea pig

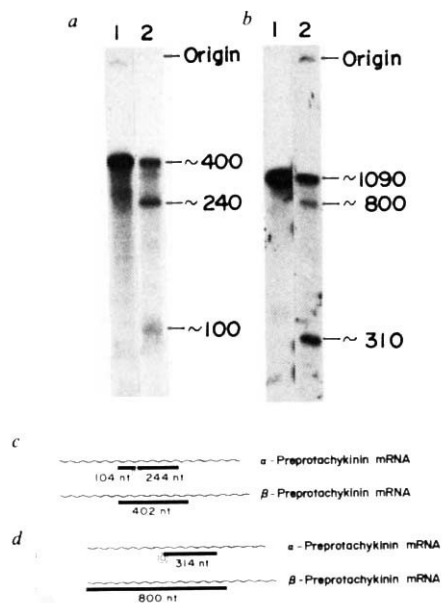


Fig. 4 Identification of two preprotachykinin mRNAs by S_1 nuclease mapping. *c, d* Predicted duplex formations between the cDNA probe and the two mRNAs; autoradiographs of *c* and *d* are shown in *a* and *b* respectively. In *c* and *d*, nucleotide numbers (nt) indicate the predicted sizes of ^{32}P -labelled cDNA fragments (thick lines) which should be protected from S_1 nuclease digestion. In *a* and *b*, lane 1 shows the cDNA probe without S_1 digestion; lane 2 shows the S_1 digestion products of the RNA-DNA hybrids. The sizes of the protected cDNA species are given in nucleotides on the right sides of autoradiographs *a* and *b*, and were estimated by comparison with the 5'-end-labelled *Hpa*II cleavage products of pBR322 (*a*) and 5'-end-labelled *Hae*III cleavage products of ΦX174 DNA (*b*).

Methods: For *a* and *c*, the 1,066-bp *Rsa*I fragment containing most of the cDNA insert was isolated from clone pSP307, nick-translated²³ with [α - ^{32}P]dCTP and cleaved with *Dde*I. The 402-bp *Dde*I fragment (residues 183–584) was then isolated and used as a probe. The probe (4×10^5 c.p.m., 2×10^8 c.p.m. per μg DNA) was denatured, hybridized to total striatal poly(A) RNA (10 μg) in 80% formamide at 41 °C for 3 h and digested with S_1 nuclease¹⁵. The products of S_1 digestion and the size marker were electrophoresed on 8.3 M urea/5% polyacrylamide gel. For *b* and *d*, the 1,089-bp *Taq*I fragment was isolated from pSP307 and labelled with ^{32}P at the 5' end. The probe used was 2×10^5 c.p.m. (3×10^6 c.p.m. per μg DNA) and the hybridization temperature was 50 °C. Other details are the same as those described for *a* and *c*.

ileum and participates in lowering blood pressure and stimulating salivary secretion. On the other hand, the SP-E receptor is activated most potently by kassinin and eledoisin and is present in the rat vas deferens and in the urogenital system of various mammals. Thus, substance K may represent a naturally occurring tachykinin responsive to the SP-E receptor and may exhibit a set of the SP-E type of biological activities in mammalian species. If this is the case, the two tachykinins liberated from β -protachykinin affect both types of receptor, while that from α -protachykinin acts only on the SP-P receptor. Thus, the differential expression of the two preprotachykinins may produce the mutually related but different physiological responses in the CNS and in peripheral tissues.

The finding of the two preprotachykinin mRNAs that simply differ by the insertion of the substance K region raises an interesting question regarding the gene(s) for preprotachykinins. As the mRNA was extracted from pooled striata from at least 10 cattle, the two nucleotide substitutions observed in the 5'- and 3'-untranslated regions of the two mRNAs may be accounted for by allelic variation. Thus, it is possible that if the inserted sequence containing the substance K sequence were specified by a discrete genomic segment, alternative RNA splicing events would ultimately generate these two mRNAs from a single

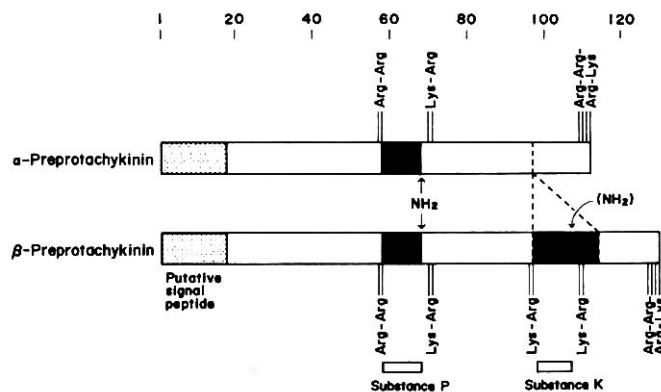


Fig. 5 Schematic representation of the structures of two bovine preprotachykinins. The sequences of substance P and substance K are indicated by the solid and lined boxes respectively, and the putative signal peptide by the dotted box; the termini of substance K and the signal peptide are not definitive. The shaded area in the β -preprotachykinin structure represents the deleted sequence of α -preprotachykinin. The two and four consecutive basic amino acid residues are all shown. NH₂ represents the site of the amidation for substance P or such a possible site for substance K. Amino acid numbers are given above the structures.

preprotachykinin gene. This model parallels the molecular events reported for the generation of multiple mRNAs in several viral genes^{17,18} as well as some cellular genes such as the calcitonin gene⁹ and the α A-crystallin gene¹⁹. Alternatively, there may be two or a set of homologous non-allelic genes for preprotachykinins, as there are for some other neuropeptide precursors such as the vasopressin and oxytocin precursors^{20,21} and the precursors containing egg-laying peptides¹⁰. Whatever the mechanism, the two preprotachykinin mRNAs have a structural relationship in which the same peptide, substance P, is released either alone or in association with a different tachykinin and thus the information potential of the gene(s) would be further increased.

We have previously elucidated the whole primary structures of the three opioid peptide precursors, ACTH- β -LPH precursor and preproenkephalins A and B, by determining the nucleotide sequences of cloned DNAs complementary to their mRNAs^{8,22,23}. The precursor proteins have multiple repetitive units, each unit containing a MSH or enkephalin sequence. The repetitive units as well as other biologically active peptides such as adrenocorticotrophic hormone and dynorphin are all bounded by paired basic amino acid residues. Thus, there is a close resemblance between the structural organizations of β -preprotachykinin and the three opioid peptide precursors and such structural organizations may have commonly occurred in neuroendocrine genes during evolution to increase the diversity of neural and hormonal peptides which may function coordinately or synergistically with one another.

Among members of the tachykinin family, substance P has so far been considered to be the only representative tachykinin in mammals. The present study indicates, however, that the kassinin-like peptide probably exists in mammalian tissues. The elucidation of the amino acid sequence of substance K should facilitate future work concerning biological activities, distribution and localization of substance K in the mammalian organism. Furthermore, it is still possible that other members of the tachykinin family may occur, either alone or in association, in different tissues and that they may subserve a variety of functions. Further unravelling of such peptides and precursors is likely to prove fruitful in understanding the physiological role of the tachykinin system in the mammalian organism.

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Optical recording of action potentials from vertebrate nerve terminals using potentiometric probes provides evidence for sodium and calcium components

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Optical methods are shown to monitor action potentials from a population of nerve terminals in the neurohypophysis of Xenopus. Calcium antagonists such as cadmium and nickel ions block a component of the action potential that probably reflects a calcium-mediated potassium conductance, and tetrodotoxin blocks an inward sodium current, revealing a calcium component to the action potential upstroke.

A COMPLETE understanding of the physiology of synaptic transmission in vertebrates has been delayed by our inability to monitor the action potential in nerve terminals. The giant synapse of the squid has served as a successful invertebrate model for the study of the release of transmitter substances^{1–3} because both the postsynaptic and presynaptic membranes are accessible for electrophysiological investigation. Much of our knowledge of vertebrate synaptic physiology is derived from the study of the frog neuromuscular junction⁴, but in this instance 'the small size of the nerve terminal prevents a direct measurement of the presynaptic potential change'⁴.

The vertebrate hypothalamo-neurohypophysial system represents an alternative model for the study of excitation–secretion coupling at nerve terminals. Magnocellular neurones located in the supraoptic and paraventricular nuclei in mammals (or in the preoptic nucleus of lower vertebrates) project their axons as bundles of fibres through the median eminence and infundibular stalk to terminate in the neurohypophysis, where the neurohypophysial peptides and proteins are secreted into the circulation. The neurohypophysis has been a classical *in vitro* preparation for measuring the calcium-dependent release of peptide hormones in various conditions of stimulation^{5,6}, and extracellularly recorded compound action potentials are readily measurable in this organ⁷. The analysis of excitation–secretion coupling in the neurohypophysis has been hindered, however, because these nerve terminals are also too small (0.5–1.0 μm) for intracellular measurement of the electrophysiological events that affect release.

We demonstrate here that optical methods that use potential-sensitive molecular probes can monitor rapid changes in mem-

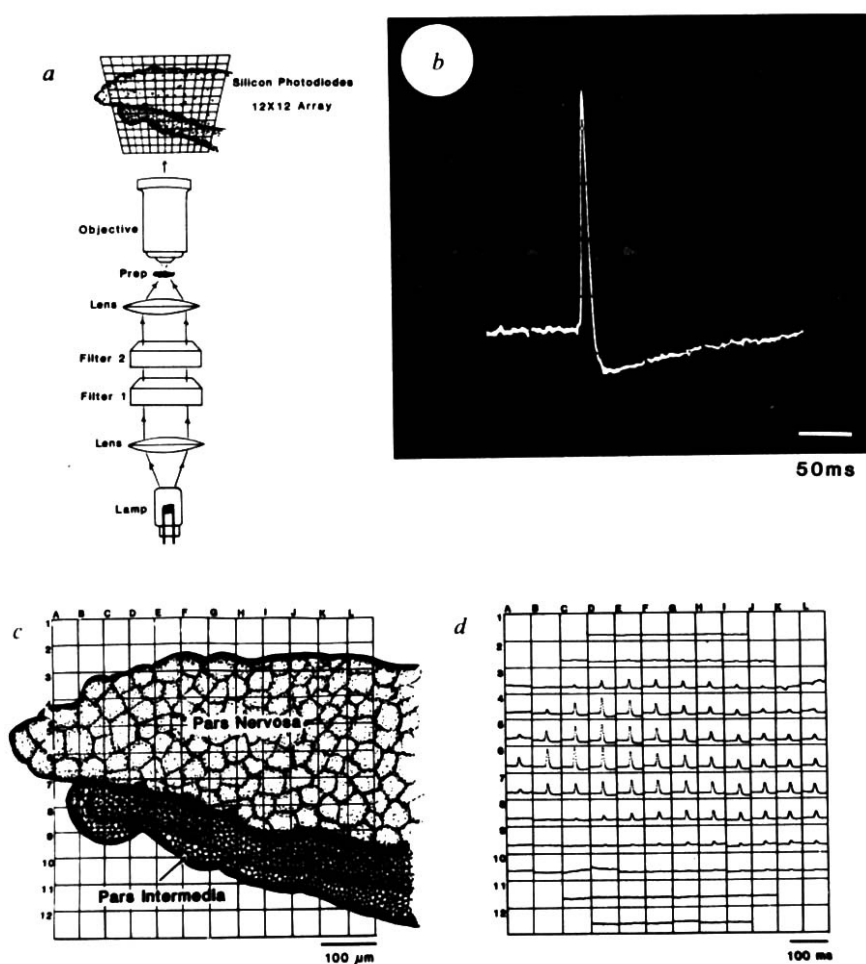
brane potential from a population of nerve terminals in the posterior pituitary (neurohypophysis). Optical methods are capable of measuring rapid changes in membrane potential^{8–10} from membranes which are inaccessible to microelectrodes. Resting and slowly changing transmembrane voltages in synaptosomes prepared from rat brain homogenates have been measured using cyanine¹¹ and merocyanine¹² dyes, and action potentials have been recorded from the growth cones of tumour cells in tissue culture¹³ using an oxonol dye. We describe here the detection of action potentials from a population of nerve terminals in the intact amphibian neurohypophysis, and the manipulation of the shape of the action potential by extracellular calcium and other agents known to alter the release of neurohormones and neurotransmitters. Calcium antagonists such as cadmium and nickel ions are shown to block a component of the action potential that probably reflects a calcium-mediated potassium conductance¹⁴, and tetrodotoxin (TTX) blocks an inward sodium current, revealing a small calcium component of the action potential upstroke.

Transmembrane voltage recording

Figure 1*b* shows an optical recording that represents the intracellular potential changes during the action potentials in a population of synchronously activated vertebrate nerve terminals in the neurohypophysis of the frog, *Xenopus laevis*. The recording was photographed from an oscilloscope screen, without signal averaging. The method that we have used for multiple-site optical recording of transmembrane voltage (MSORTV) is illustrated to the left of the record (Fig. 1*a*). In the experiment illustrated in Fig. 1, part of the neurointermediate lobe was

Fig. 1 Multiple-site optical recording of transmembrane voltage (MSORTV) in neurohypophysis of *Xenopus*. **a**, Schematic diagram of the optical portion of the MSORTV system. Collimated light from an incandescent source is rendered quasi-monochromatic with interference and heat filters and focused on the preparation using a bright-field condenser with numerical aperture (n.a.) matched to that of the objective. A high n.a. water immersion objective projects a real image of a portion of the preparation onto the central 124 elements of a 144-element photodiode matrix array, whose outputs are converted to voltages, a.c.-coupled and amplified, multiplexed, digitized and stored in a PDP 11/34A under direct memory access (DMA). A full frame is recorded every 0.8 ms. **b**, A photograph of an oscilloscope recording of the change in transmitted intensity monitored by one channel (element E5) of the MSORTV system, following a single brief shock to the hypothalamus. This element monitored intensity changes from a region of the posterior pituitary entirely within the pars nervosa. The fractional change in intensity during the action potential was $\sim 0.25\%$. Single sweep, 722 ± 21 nm. Response time of 1.1 ms (10–90%). a.c. coupling time constant 1 s. **c**, Drawing of the region of the posterior pituitary imaged on the photodiode matrix array, showing the positions of the individual detector elements with respect to the tissue. Drawn from a photomicrograph of the preparation and a transparent overlay representing the detector array. Objective, $\times 20$, 0.33 n.a. **d**, Extrinsic absorption changes obtained in a single sweep, following stimulation of the hypothalamus, superimposed on corresponding elements of the photodiode matrix array. The five elements in each corner of the array were not connected. The largest signals represent fractional changes in transmitted intensity of $\sim 0.3\%$.

Methods: Light from a tungsten-halogen lamp was collimated, rendered quasi-monochromatic with a heat filter (KG-1, Schott Optical Company, Duryea, Pennsylvania) and an interference filter (700 nm; 70 nm full width at half maximum), and focused, by means of a bright-field condenser, on the pars nervosa of the posterior pituitary, excised together with the hypothalamus and infundibulum. The preparation had previously been vitally stained by incubating it for 25 min in a $100 \mu\text{g ml}^{-1}$ solution of the merocyanine-rhodanine dye NK 2761 (refs 32, 33), in *Xenopus* Ringer's solution (112 mM NaCl; 2 mM KCl; 2 mM CaCl_2 ; 15 mM HEPES; 6 mg ml^{-1} glucose; pH adjusted to 7.35). Transmitted light was collected by a high numerical aperture water immersion objective, which formed a real image on a 12×12 element silicon photodiode matrix array (MD 144-O; Integrated Photomatrix Inc., Mountainside, New Jersey) located in the image plane of a compound microscope (UEM, Zeiss). The photocurrents generated by the central 124-array elements were separately converted to voltages and amplified as described previously by Salzberg *et al.*³⁴ and Grinvald *et al.*³⁵. In most experiments, all amplifier outputs were fed into a data acquisition system based on a PDP 11/34A computer (Digital Equipment Corp., Maynard, Massachusetts) capable of acquiring a complete frame every 0.8 ms. This data acquisition system is similar to that described previously by Grinvald *et al.*³⁵ and used by Grinvald *et al.*³⁶ and Senseman *et al.*³⁷. In some experiments, the temporal resolution was improved when selected outputs of the current-to-voltage converters were also passed in parallel to a 16-channel signal averager (TN 1500; Tracor Northern Inc., Middleton, Wisconsin); these digitized output signals were stored on magnetic tape for display and analysis. A map of the preparation was obtained by superimposing a transparent overlay representing the photodiode array elements on a photograph taken through the trinocular tube of the microscope after removing the array housing.



imaged onto a photodiode matrix array using a $\times 20$, 0.33 numerical aperture (n.a.) water immersion objective (Nikon) and a drawing of the gland (Fig. 1c) was prepared from the photograph. Figure 1d shows the MSORTV display of the action potentials recorded optically, in a single sweep, from different regions of the pars nervosa, following a single stimulus applied to the hypothalamus. The optical signals are confined to regions of the grid that correspond to the neurohypophysis; note that a small portion of the neurohypophysis lies under the pars intermedia (row 9). The optical signal shown in Fig. 1b is a photograph of an oscilloscope recording of the output from channel E5; this recording demonstrates the large signal-to-noise ratio achievable in these experiments. Control experiments¹⁵ demonstrated that these signals represent voltage changes; no signals were recorded in white light or in the absence of illumination; the signals could be inverted at shorter wavelengths.

Optical signals from nerve terminals

Evidence that the optical signals shown in Fig. 1 do effectively represent the membrane potential changes associated with action potentials in the nerve terminals of the neurohypophysis is provided indirectly by morphometric observations. In the rat, it has been estimated that the 18,000 magnocellular neurones in the hypothalamus give rise to approximately 40,000,000 terminal endings in the pars nervosa of the posterior pituitary and morphometric data suggest that here, secretory endings and swellings comprise 99% of the excitable membrane area, with axons making up the remaining 1% (ref. 16). There are no comparably detailed morphometric studies for the neurohypophysis of *Xenopus*, but the ultrastructural resemblance of the amphibian neurohypophysis to that of the rat^{17–19} suggests that, in *Xenopus*, it is also heavily enriched with nerve terminals. Therefore, we infer that in Fig. 1 the optical signals from the

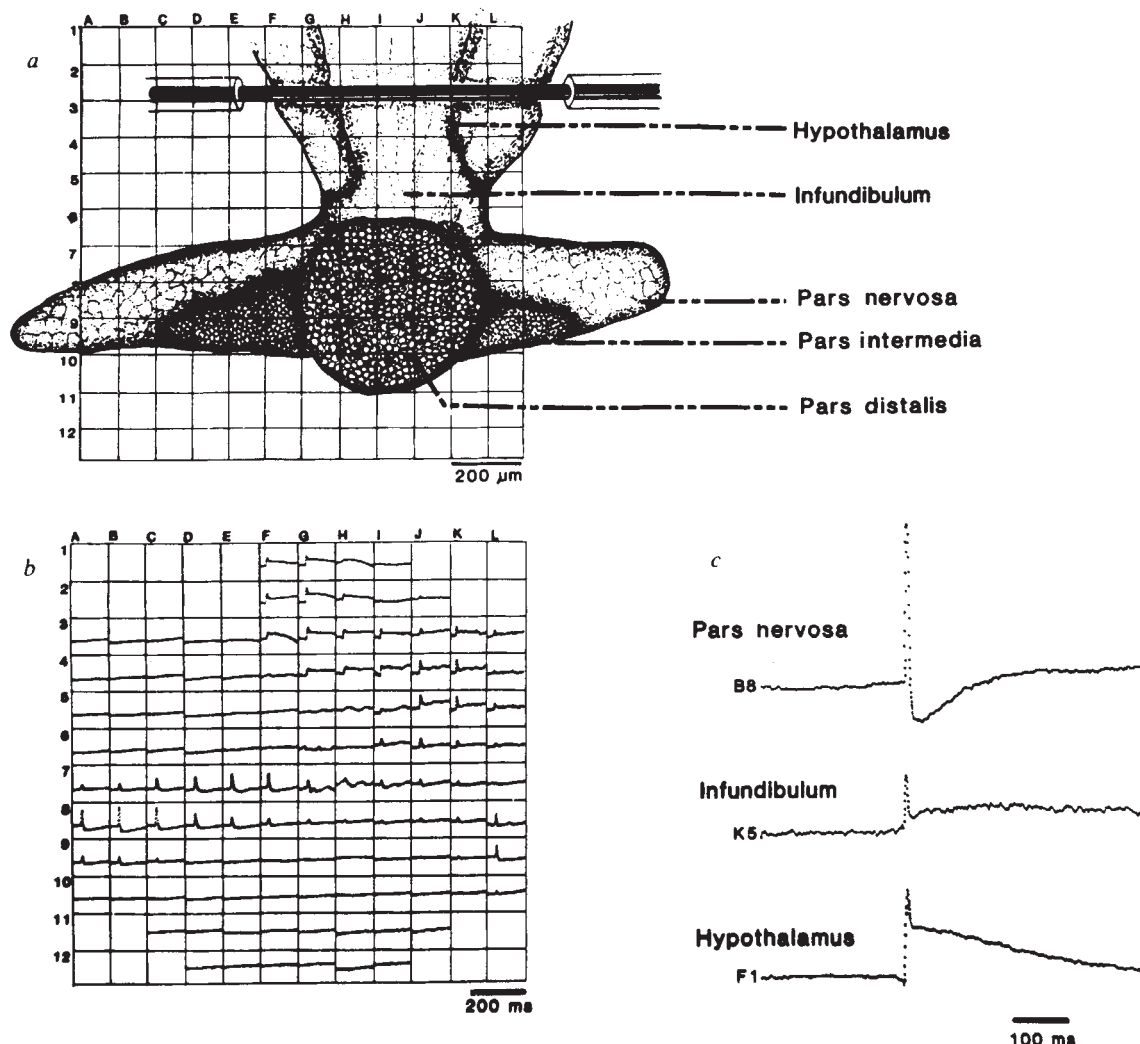


Fig. 2 Regional differences in the optical signals recorded from the hypothalamus, infundibulum and pituitary. *a*, A preparation consisting of the hypothalamus together with the entire pituitary gland, shown superimposed on a grid representing the photodiode matrix array. One of two stimulating electrodes, near the level of the median eminence, is shown. Objective, $\times 10$, 0.25 n.a. *b*, Extrinsic absorption changes, recorded in a single sweep, shown superimposed on the elements of the photodiode matrix array corresponding to the data channels from which they were obtained. Wavelength 722 ± 21 nm. *c*, Enlargements of the optical signals characteristic of three different regions of the preparation. The coordinates of the matrix array element from which the signal was obtained are shown at the beginning of each trace.

lateral portions of the pars nervosa are dominated by the membrane potential changes in the magnocellular neurone terminals.

Further evidence for the origin of the optical signals is shown in Fig. 2. In this experiment, the use of a lower magnification ($\times 10$; 0.25 n.a.) permitted optical recording from a larger region of the preparation that included the neurointermediate lobe (pars nervosa and pars intermedia), infundibular stalk, anterior pituitary (adenohypophysis or pars distalis) and a portion of the hypothalamus. Figure 2*a* shows a drawing of the preparation (made from a photomicrograph) and illustrates the mode of stimulation. A pair of fine Teflon-coated silver electrodes, having 600 μm long bare regions, clasp the hypothalamus at the level of the median eminence and serve to immobilize the preparation. The electrodes deliver brief (0.5 ms) stimulating shocks to the hypothalamus, on command from the computer. The grid superimposed on the drawing indicates the size and positions of the photodiode array elements; the grid reproduced in Fig. 2*b* displays, on corresponding elements, the absorption signals measured simultaneously in a single sweep. Figure 2*c* shows, enlarged, representative signals from three regions. The top trace (from element B8) shows a typical action potential recorded from a site entirely within the neural lobe (pars nervosa), where the nerve terminals are dominant. The middle trace (from element K5) shows the optical signal from the infundibular stalk where there is a relatively pure population of axons of the

magnocellular neurones, and few terminals. The lower trace (from element F1) shows the optical signal recorded from the hypothalamus, proximal to the stimulating electrodes.

There is a marked difference, in both size and shape, between the signals recorded from the neurohypophysis and infundibulum. The former are relatively large and brief, with a prominent afterhyperpolarization. The infundibular spike is small and is followed by a long-lasting depolarization that may have a non-axonal origin. If this slow signal is non-axonal, the axonal signal is even smaller, compared with the action potential from the pars nervosa. If the optical spike recorded from the neurohypophysis (B8) arises from the numerous nerve terminals that are likely to comprise the overwhelming proportion of excitable membrane in this region¹⁷⁻¹⁹, the difference in size of these optical signals would be expected on the basis of membrane areas: the membrane area per unit volume is far greater in the neurohypophysis than in the infundibulum. The difference in signal size represents a variation in fractional intensity change because the resting transmitted intensities from the two regions are comparable. If the waveform recorded from the infundibulum approximates the true shape of the action potential in infundibular axons, the different shape of the optical signal obtained from the pars nervosa strongly suggests that nerve terminals rather than axons are the origin of the signals derived from the neurohypophysis.

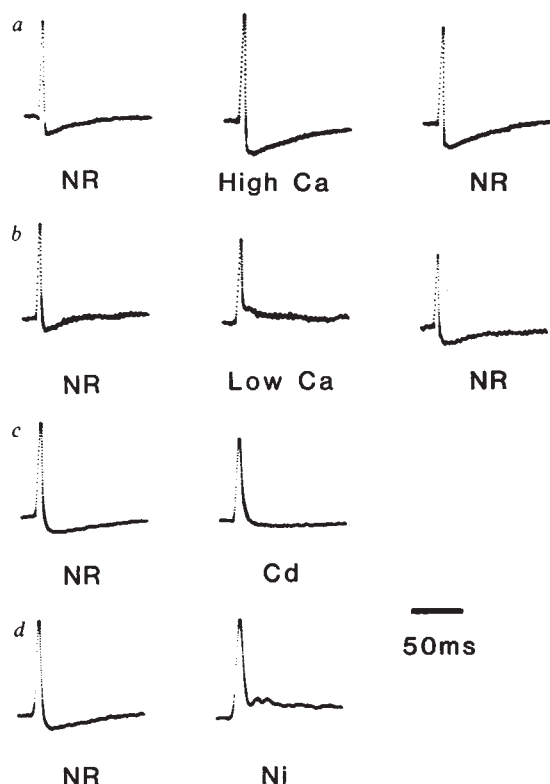


Fig. 3 Calcium dependence of the afterhyperpolarization of the action potential recorded from nerve terminals in the neurohypophysis. *a*, Effect of elevated Ca^{2+} on the nerve terminal action potential. Left-hand trace shows the nerve terminal action potential, recorded in normal Ringer's solution (NR; 2 mM Ca^{2+}). This, and all records that follow, were obtained in single sweeps from an element of the photodiode array monitoring potential changes from nerve terminals in the neurohypophysis. The action potential shown in the middle trace (High Ca) was recorded after 10 min in a Ringer's solution containing 5 mM Ca^{2+} . The right-hand trace (NR) shows the nerve terminal action potential 10 min following the return to normal (2 mM Ca^{2+}) Ringer's solution. *b*, Effect of lowered extracellular Ca^{2+} on the nerve terminal action potential. The left-hand trace (NR) shows the control action potential. The action potential in the middle trace (Low Ca) was recorded 10 min after replacement of the bathing solution with one containing 0.1 mM Ca^{2+} and 2 mM Mg^{2+} . The right-hand trace (NR) shows the nerve terminal action potential 10 min following return to control (2 mM Ca^{2+}) Ringer's solution. Some bleaching is evident from the reduction in overall signal size. *c*, Effect of 0.2 mM Cd^{2+} on the shape of the nerve terminal action potential. Left-hand trace (NR) shows the action potential in normal Ringer's solution. Right-hand trace (Cd) shows the action potential 2 min following the addition of 0.2 mM Cd^{2+} to the normal Ringer's solution. This effect could not be reversed. *d*, Effect of 1 mM Ni^{2+} on the shape of the nerve terminal action potential. Left-hand trace (NR) shows the action potential in normal Ringer's solution. Right-hand trace (Ni) shows the action potential 2 min after the addition of 1 mM Ni^{2+} to normal Ringer's solution. This effect could not be fully reversed.

The signals recorded from the hypothalamus (for example, Fig. 2c, F1) have an entirely different shape, with a slowly decaying depolarization. These signals may represent a weighted average of hypothalamic neuronal spikes, but they may also be complicated by potential changes in glial cells or recurrent hypothalamic neuronal activity, or both.

Properties of the action potential

The nerve terminal action potentials shown in Fig 1 and 2 exhibit two unexpected features. First, they have relatively long durations (that is, they have a full width at half height of ~6 ms) compared with those recorded from vertebrate axons (for example <2 ms in the frog)²⁰. However, there must be some temporal dispersion in the population, and this represents an

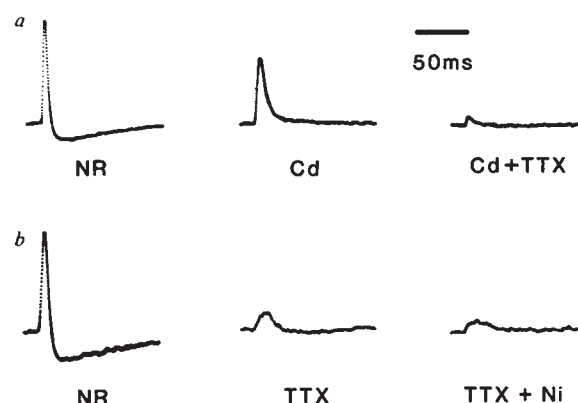


Fig. 4 Effects of sodium and calcium channel blockers on the rising phase of the nerve terminal action potential elicited by field stimulation of the neurohypophysis. *a*, Left-hand trace (NR) shows the nerve terminal action potential in normal Ringer's solution. The middle trace (Cd) shows the action potential 18 min after the addition of 0.2 mM Cd^{2+} to the normal Ringer's solution. The right-hand trace (Cd+TTX) shows the optical signal recorded from the same population of nerve terminals 6 min after the addition of 5 μM TTX to normal Ringer's solution containing 0.2 mM Cd^{2+} . *b*, Left-hand trace (NR) shows the nerve terminal action potential in normal Ringer's solution. The middle trace (TTX) shows the residual potential change after 10 min exposure to 5 μM TTX in normal Ringer's solution. The right-hand trace (TTX+Ni) shows the further reduction in size of the signal on addition of 2 mM Ni^{2+} to normal Ringer's solution containing 5 μM TTX. Nickel may block only part of the TTX-resistant response.

upper bound on the spike duration. This value is in good agreement with the 5–6-ms duration reported for the action potentials of magnocellular neurone somata²¹ and the compound action potential in the neurohypophysis⁷. The second characteristic of the nerve terminal action potential is its conspicuous afterhyperpolarization that is reminiscent of the afterhyperpolarization frequently associated with a calcium-mediated potassium conductance (for example, in snail neurones¹⁴, rat myotube²², guinea pig Purkinje cells^{23,24}).

We found that we could alter the afterhyperpolarization recorded optically by changing the ionic composition of the bath. Figure 3 shows the results of a preliminary series of experiments designed to examine the calcium dependence of this long-lasting hyperpolarization. The traces shown represent typical outputs from single channels of the 124-channel MSORTV system; each trace is a single sweep. In each row, the optical signal on the left (NR) was recorded in a control Ringer's solution containing 2 mM Ca^{2+} ; these signals are normalized to the same peak height. The middle trace in Fig. 3a (High Ca) shows the effect of a 10-min exposure to elevated (5 mM) extracellular Ca^{2+} concentration: the afterhyperpolarization increased by ~90%. This is a lower limit, because dye is bleached during each measurement and some diminution of the signal is inevitable. We always found a large increase in the size of the afterhyperpolarization, although the magnitude of the effect varied widely. An increase in the height of the peak (14% in Fig. 3a) was often observed; this finding suggests that an inward Ca^{2+} current might contribute to the rising phase of the action potential (see below). The record on the right in Fig. 3a (NR) shows the optical signal from the same region of the neurohypophysis 10 min after the return to 2 mM Ca^{2+} .

The optical traces in Fig. 3b show the effect of lowering extracellular Ca^{2+} to 0.1 mM (by replacing the Ca^{2+} with Mg^{2+}). The middle trace (Low Ca) shows that after 10 min the undershoot is completely absent, replaced by a long-lasting depolarization. The magnitude of this effect also varied between preparations; however, a reduction of external calcium to ≤ 0.1 mM never failed to reduce the afterhyperpolarization. Further lowering of extracellular calcium occasionally eliminated the action potential altogether, but we believe that this probably resulted

from a steady-state depolarization to which the optical measurements are insensitive. The right-hand trace in Fig. 3b (NR) illustrates the recovery (10 min back in 2 mM Ca^{2+}). In this experiment, reduction of the signal due to bleaching was more apparent. While the effects of changes in extracellular calcium were always reversible, this was not the case with other ionic substitutions. Cadmium ions are known to block calcium currents in several preparations (see for example, ref. 25) and Fig. 3c shows that after 2 min in 200 μM Cd^{2+} (right-hand trace; Cd), the afterhyperpolarization was almost entirely eliminated. This effect was only partially reversible. Longer exposure to cadmium eliminated the afterhyperpolarization completely. The peak height was also decreased, but, in the absence of recovery, it is difficult to know whether this is an effect of bleaching. Figure 3d shows that Ni^{2+} , another inorganic calcium blocker²⁶, at millimolar concentrations also blocks the spike afterhyperpolarization. In some experiments, nickel produced a small but significant increase in the peak height. The origin of this effect is unclear, although a change in resting potential has not been ruled out. All the experiments in Fig. 3 suggest that calcium has a role in shaping the action potential in the vertebrate nerve terminals studied here. Preliminary experiments with other calcium antagonists such as Mn^{2+} and Co^{2+} are consistent with this observation, although they seem to be less potent than Cd^{2+} .

Ionic basis of depolarizing phase

Because calcium entry seems to play a part in excitation-secretion coupling, we attempted to determine whether an inward calcium current contributed measurably to the depolarizing phase of the action potential. *A priori*, calcium is unlikely to be the dominant carrier of the inward current in the nerve terminals of the neurohypophysis, because the surface-to-volume ratio in these small secretory endings would result in the imposition of a large, probably intolerable, calcium load. Figure 4 illustrates an experiment designed to examine this point. Inasmuch as calcium antagonists leave the depolarizing phase of the action potential largely intact, sodium may be carrying the bulk of the inward current. If so, it may be blocked by TTX, and a positive result would provide evidence that the rising phase of the action potential is due largely to the activity of voltage-dependent sodium channels. To test this possibility it was necessary to change the stimulation conditions, because, in the presence of TTX, blockade of the sodium action potential in the axons of the infundibulum would prevent the excitation of the nerve terminals in the neurohypophysis. We therefore applied a form of field stimulation in which the lateral tips of the pars nervosa were held by suction electrodes, and very brief (0.2 ms) currents were passed between the two electrodes. In this way, the terminals could be excited directly, without the need for axonal transmission. This technique was used in the experiment shown in Fig. 4. The trace on the left in Fig. 4a (NR) shows the control, in 2 mM Ca^{2+} Ringer's solution. The middle trace (Cd) was recorded from the same population of

nerve terminals following 18 min exposure to 200 μM Cd^{2+} , to block the inward calcium current. The reduction in the magnitude of the action potential is obvious, together with a considerable broadening, and the complete loss of the afterhyperpolarization. The extent of this reduction, however, may be exaggerated by factors other than blockade of calcium channels (for example, dye bleaching). The right-hand trace in Fig. 4a ($\text{Cd} + \text{TTX}$) shows that 6 min after application of 5 μM TTX, the Cd^{2+} -resistant portion of the terminal action potential was completely eliminated. The residual signal seen in this record is the passive depolarization produced by the field stimulation; it was reversed with reversal of stimulus polarity, and exhibited the same wavelength dependence as that of the optical recording of the action potential.

The complementary experiment was also carried out (Fig. 4b), in which the sodium current was blocked first with TTX (Fig. 4b, middle trace, TTX) and the size of the small residual 'spike' was decreased substantially by both Ni^{2+} (right-hand trace, $\text{TTX} + \text{Ni}$) and Cd^{2+} . Thus, the experiments shown in Fig. 4 suggest that in the neurohypophysis of *Xenopus*, the rising phase of the nerve terminal action potential is mediated predominantly by sodium, although the data suggest at least a small calcium component. The magnitude of this component is difficult to assess quantitatively because the TTX blockade of the normal regenerative sodium depolarization may limit the opening of voltage-dependent calcium channels. The presence of a small calcium component in the largely sodium-dependent action potential in rat pars intermedia has been reported recently by Douglas and Taraskevich²⁷ and a similar situation obtains in fish pars distalis (prolactin) cells²⁸. The important role of calcium in excitation-secretion coupling is fully consistent with its apparently small contribution to the terminal action potential²⁹. In adrenal chromaffin cells, where calcium influx is the critical event in excitation-secretion coupling³⁰, the Ca^{2+} component of the action potential is also small³¹.

Conclusions

The anatomy of the posterior pituitary confers two advantages for studies *in vitro* of excitation-secretion coupling: there is no postsynaptic excitable membrane to confound the interpretation of the optical signals and no barrier to the collection of its secretory products. We expect that direct optical measurement of transmembrane potential changes from the nerve terminals of the anuran and the mammalian neurohypophysis, when correlated with secretion of neurohypophysial peptides, will provide new insight into the electrophysiology of transmitter and hormone release in the vertebrates.

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A rapid millimetre wave outburst in the nucleus of NGC1275

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A very rapid large-amplitude outburst was observed in the Seyfert-like galaxy NGC1275 at millimetre wavelengths but not at lower radio frequencies. At 89.6 GHz, the flux density rose from 42 Jy to 75 Jy between 26 October 1979 and 13 March 1980 at a rate reaching 140 Jy yr^{-1} . The event was also evident at 1 mm wavelength, where the flux density increased from 24 Jy to 64 Jy between measurements made on 11 Jan 1979 and on 28 March 1980¹. Observations at 31.4 GHz limit any simultaneous outburst to 20 Jy or less. This is the first time a strong outburst has been seen at millimetre but not centimetre wavelengths in any radio source.

The Seyfert-like galaxy NGC1275 (3C84 or 0316+41) was the first radio source found to have an inverted radio spectrum² and was also one of the first sources known to be variable at centimetre wavelengths³. Since 1960 its centimetre-wavelength emission has risen steadily. Its millimetre-wavelength emission has shown minor fluctuations about a constant level since 1965⁴⁻⁷.

Our flux density measurements at 89.6 and 31.4 GHz are shown in Fig. 1. The majority were made with the 36-foot antenna of the National Radio Astronomy Observatory using a dual-beam position switching technique. The flux densities were calibrated relative to DR21 which was assumed to have a flux density of 16.9 Jy at 89.6 GHz and 18.4 Jy at 31.4 GHz^{8,9}. We have also included additional 3-mm measurements made between 1979 and 1981 with the Five College Radio Astronomy Observatory 14-m antenna at the University of Massachusetts¹⁰. These measurements have improved the sampling and helped to define the rapid changes in NGC1275.

The 1980 outburst in NGC1275 is also evident in the 31 GHz measurements, however the outburst at this frequency was delayed by 0.7 yr and its amplitude was 7 Jy compared with 33 Jy at 89 GHz. The amplitude at 15 GHz was $<1 \text{ Jy}$ ¹⁴. The 89 and 31 GHz data also show that the strong 1980 outburst was preceded by a weaker 9 Jy outburst in 1978. In contrast to what was observed in the major outburst, this precursor exhibited similar amplitudes at both 89 and 31 GHz and was also delayed (0.4 yr) at the lower frequency.

About one year after the onset of the 1980 outburst, the 89 GHz flux density dropped to a level midway between its peak and pre-outburst levels. The decline, however, was not continuous. There appeared to be a drop to a new level, a recovery to the previous level, and finally a drop again to the same level. The time scale of this fluctuation was $\leq 0.2 \text{ yr}$ since our measurements did not adequately sample the event. The sparse 300 GHz data¹ suggest that the outburst decayed earlier at this frequency while our data show that there is no evidence for a decay at 31 GHz.

The progressive delay of an outburst at lower frequencies implies that the source opacity (τ_ν) at frequency ν in the region of synchrotron emission is decreasing with time. For electrons with a power law energy distribution of index γ , the opacity

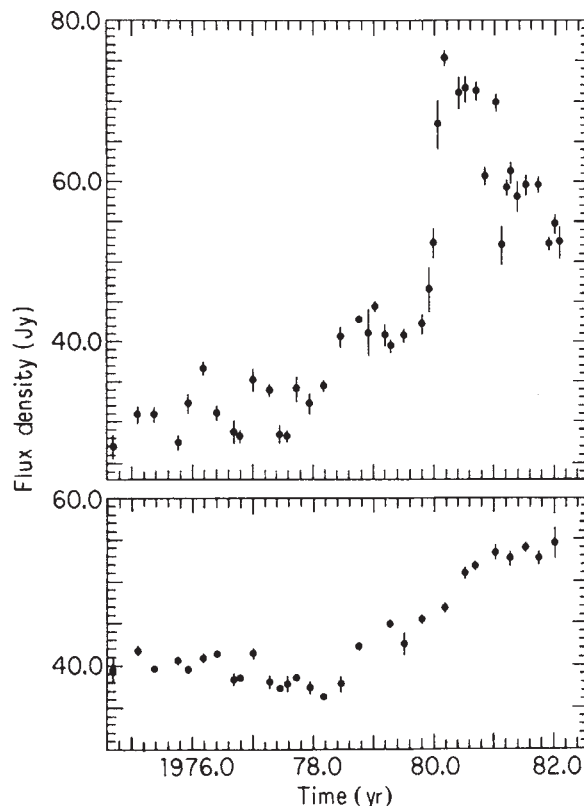


Fig. 1 Flux density variation in 3C84 at 89.6 (top) and 31.4 GHz (bottom). The measurements have been averaged over a time interval of 1 week.

due to synchrotron self-absorption is¹⁸:

$$\tau_\nu(t) \propto N(t)R(t)B(t)^{(\gamma+2)/2} \nu^{-(\gamma+4)/2}$$

The delay at frequency ν can be explained by a decrease in the density of relativistic electrons N or the strength of the magnetic field B , or both. In the simple expanding source model¹¹ both N and B decrease adiabatically as the radius of the emitting region R increases. The difference in amplitude from one outburst to another is simply due to a difference in the number of electrons accelerated to relativistic speeds.

The evolution of the radio spectrum of the 1980 outburst can be obtained by subtracting the contributions of other radio components unresolved by the antenna beam. Milliarc second resolution maps¹² have shown that the centimetre-wave structure of the nucleus of NGC1275 is very complex consisting of three colinear components 'North, Centre, and South' (N , C , and S respectively), plus many weaker components. A model for the 1980 spectra of these centimetre-wave components, based upon the 5.0–10.7 GHz maps of Unwin *et al.*¹³, was subtracted from our measurements of the integrated spectrum between 2.7 and 89.6 GHz. An iterative least-squares technique was used to solve for the millimetre spectral components¹⁴. Figure 2 shows the 'deconvolved' radio spectrum in 1980 at the time of the large outburst.

In addition to the three major centimetre-wave components (N , C and S), the least-squares fit requires the existence of a millimetre component, M , whose spectrum peaks near 50 GHz. This component has dominated the emission at millimetre-wavelengths over at least the past decade. A high resolution 22 GHz map made in 1981¹⁵ shows that the North component consists of two components (A and B). The northernmost component (A) had a flux density of 6.5 Jy and a positive spectral index (slope) at 22 GHz. It is probable that ' A ' is the site of our spectral component M , while our spectral component N is consistent with the less compact component B ¹⁴.

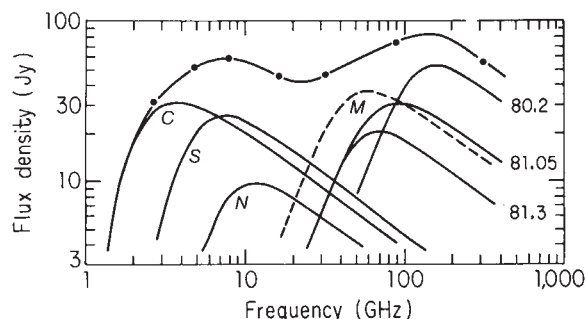


Fig. 2 The decomposition of the total flux density spectrum of 3C84 at three epochs during the large millimetre outburst. See ref. 14 for description of the deconvolution procedure. The lower frequency components (C, S, N) did not change appreciably during this time interval.

The spectrum of the outburst at three epochs is also shown in Fig. 2. The spectral peak evolved from ~ 150 GHz at the outburst maximum in March 1980 to ~ 70 Jy 1.1 yr later, as the peak flux density dropped from ~ 53 Jy to ~ 20 Jy by April 1981. This evolution is not continuous but jumps abruptly from one spectrum to the next. Before 1981.0 the 31 GHz emission is rising, implying that the source was expanding. However, after 1 January 1981 the 31 GHz flux density remained constant probably because the outburst ceased expanding. Thus, the later decay of the 89 and 300 GHz emission is not due to expansion but is most probably due to a reduction in particle acceleration coupled with an efficient high energy loss mechanism. The fluctuations observed before dropping to the lower level could be due to an intermittency in the production of relativistic particles. This interpretation would imply that the major outburst itself is a superposition of pulses blended together by a rapid repetition rate. When the rate is reduced as the source energy is diminished, the individual pulses would be discernible as suggested by the fluctuations around 1 January 1981 at 89 GHz.

The rapid onset of the outburst and the short time scale fluctuations during the decay at 89 GHz imply that the size of the region in which the radiating particles are accelerated is less than $c\tau_{\text{rise}}$ or 10^{17} cm (assuming nonrelativistic expansion). Adopting this size, the computed brightness temperature of the outburst at maximum is 10^{12} K, right at the limit where inverse Compton collisions quench the synchrotron emission¹⁶. Inverse Compton quenching would explain the rapid drops in emission and may explain the relatively flat peak of the outburst as it would place an abrupt limit on the luminosity of synchrotron emission within the volume of the core. If this is the case, there should have been an optical or X-ray outburst occurring nearly simultaneous with the millimetre-wave outburst.

The first milliarc second resolution observations at short millimetre wavelengths were made by Readhead *et al.*¹⁷ at 22 October 1981, while the millimetre outburst in NGC1275 was decaying. Their preferred interpretation attributes 19 Jy to the flux density of the very compact 'A' component at the north end of the jet. Our temporal measurements show that the total flux density at 89 GHz was 60 Jy at this time. The difference between this level and the pre-outburst level of 40 Jy due to other components agrees well with the 19 Jy 'A' component at the end of the jet. The measured dimensions of this component¹⁷ are 2 by 15×10^{17} cm. If this component began expanding uniformly at the onset of the 1980 outburst, the large dimension above implies an expansion velocity that was $\leq 0.9 c$.

Our results suggest that the large-amplitude millimetre outburst which occurred in the energetic core is located at the northernmost end of the jet and that it and other outbursts supply relativistic particles whose synchrotron emission is later observed at lower frequencies farther on down the jet.

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Non-radial ejection in 3C345

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The radio source 3C345 is associated with a 16th magnitude quasar with $z = 0.595$ (ref. 1). At radio wavelengths it has six major components. From largest to smallest these are: a low brightness halo²; a 3 kpc curved jet³; three steep-spectrum components (C_1 , C_2 , and C_3) which define a curved jet^{4,5}; and a compact component D which is self-absorbed at frequencies up to at least 10 GHz. Components C_2 and C_3 show proper motion relative to D at an apparent superluminal speed. We present VLBI observations at 10.7 GHz which show that a new radio component C_4 has appeared and is moving along a non-radial path. This behaviour is consistent with the hypothesis that bending causes the jet curvature. We interpret our observations in light of the relativistic beam model⁶ as this is currently the most attractive explanation for both the superluminal effect and the weak X-ray emission⁴.

The position angle (PA) misalignment between the small- and large-scale structures, which is seen in many radio sources, is especially large in 3C345 (ref. 7). Observations at 22 GHz show that the PA of the structure at radius (r) ~ 0.3 m arc s is about -130° (refs 7, 8) while the large scale jet ($r \sim 3$ arc s) has PA $\sim -30^\circ$ (ref. 3). Explanations of this misalignment or curvature include motion along a bent jet⁹ or precession of the central engine combined with ballistic motion of the ejecta¹⁰. In both cases the curvature could be strongly amplified by projection, so the large observed misalignment is plausible. Cohen *et al.*⁵ have recently presented evidence that component C_2 moved radially at a constant PA from $r = 1$ to 4 m arc s during the period 1971–82, which is consistent with the precession hypothesis. We now present evidence, however, that the motion of a new component C_4 is non-radial with respect to D. The new evidence is consistent with motion along a bent jet. We return to this apparent discrepancy below.

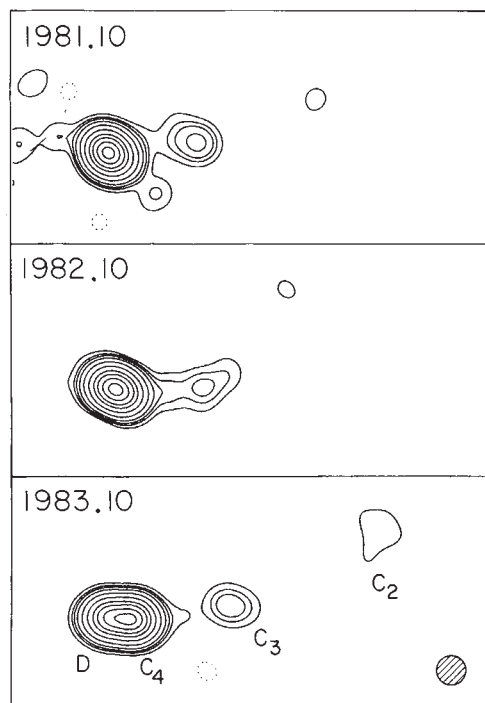


Fig. 1 Contour plot of clean map from three epochs. Contour levels are at $-1, 1, 2, 3, 5, 10, 20, 35, 50, 70$, and 90% of the peak intensity of $6.9, 9.4$, and 7.7 Jy per beam area respectively at the 1981.1, 1982.1, and 1983.1 epochs. The hatched region indicates the clean restoring beam, which is a 0.50 m arc s circular gaussian function. North is at the top, and the width of the map is 10.00 m arc s.

VLBI observations were made at four epochs between 1981.1 and 1983.1 at $10,651$ MHz in left-circular polarization with a bandwidth of 1.8 MHz. At each epoch the longest baseline gave an angular resolution $\lambda/2D = 0.33$ m arc s. Details of the observations will be presented elsewhere.

Hybrid maps were made for all except the third epoch; the mapping technique described by Unwin *et al.*⁴ was used. The maps are presented in Fig. 1. For each epoch, two-component models were fit to the bright eastern region which had a scale size <1 m arc s. These models consisted of an eastern point source and a western elliptical gaussian component. This model gave a much better fit than a pair of point sources. A least-squares algorithm was used to obtain model parameters which best fit the visibility amplitudes and closure phases. In all cases the final model was insensitive to the starting model.

Figure 1 shows that the source consists of a slightly resolved eastern region which contains most of the flux, and a faint extension ~ 2 m arc s to the west. Two-component models of the bright eastern region for all four epochs are presented in Table 1. For the assumed model the quoted uncertainties include the range of possible parameter values. Our results are in good agreement with those of Moore *et al.*¹¹ which were obtained independently at a different frequency. Minor differences between the results, as well as size and spectral evolution of the components will be described elsewhere (refs 4, 12, and J.A.B. *et al.* in preparation). For a deceleration parameter $q_0 = 0.05$ the scale is 7.9 pc m arc s⁻¹ k^{-1} where $k = H_0/55$ and H_0 is the Hubble constant.

The results may be summarized as follows:

- (1) At each epoch a minimum of two components is required to fit the bright eastern region. The models in Table 1 give a very good fit to the data.
- (2) The relative PA and the separation of the two components increase monotonically with epoch.
- (3) For epochs with maps, the PA and separation indicated by the map are consistent with the model.

As the structure of the source is not well resolved by our

Table 1 Two component models of bright eastern region in 3C345

Epoch	1981.10	1982.10	1982.86	1983.10
No. of stations	5	6	3	6
Component flux (Jy)				
eastern	5.8	6.0	5.0	4.6
western	2.8	6.4	8.4	8.1
Radius* (m arc s)	0.32 ± 0.04	0.35 ± 0.02	0.49 ± 0.06	0.53 ± 0.02
PA (deg)*	-132 ± 6	-115 ± 2	-97 ± 6	-94 ± 4

* Vector from eastern to western component.

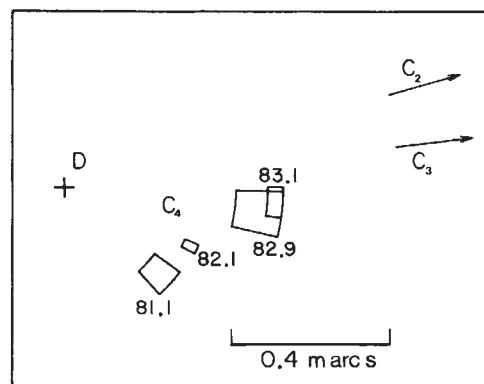


Fig. 2 Positions of component C_4 assuming core component D is stationary. North is up.

maps it must be inferred from the above models and other evidence. The structure at previous epochs consisted of an unresolved component D and discrete jet components which moved away from D (ref. 4). Unless the basic nature of the source has changed, the elongation in our maps must be due to a new jet component. Hence we identify the western component in our models with a new jet component C_4 and the eastern component with the unresolved component D. Figure 2 shows the locations of C_4 relative to D.

Our observations show that the radius vector from D to the new component has changed PA by $\sim 38^\circ$ as the component separated from D. These and similar observations presented by Moore *et al.*¹¹ are the first observation of an extragalactic radio source in which a discrete component has changed PA. In the relativistic jet model of the superluminal effect⁶ the unresolved, flat spectrum component D is near the central engine and the outer components are identified with plasma clouds or shock waves which move along a jet at relativistic velocities. The change in PA of this new component would then indicate that it has moved along a bent or curved jet. This is consistent with the suggestion⁹ that the large misalignment or curvature seen in some sources is due to projection effects which amplify a small intrinsic bend caused by external pressures. Figure 2 shows that the trajectory of the new component is not appreciably curved between $r \sim 0.3$ and 0.5 m arc s. Hence there must be a strong curvature at $r < 0.3$ m arc s if the component is to originate in the core.

Observations at 22 GHz also give evidence for this new component. In 1977.8 and 1978.7 the core was extended along PA $= -135^\circ$ (ref. 8) and in 1981.25 the extension was ~ 0.3 m arc s at PA $= -130^\circ \pm 5^\circ$ (ref. 7). These data and our own at 1981.10 (Table 1) are consistent with the hypothesis that 3C345 had a slowly moving component with proper motion $|\mu| < 0.1$ m arc s yr⁻¹ for 3 yr or more. For the data in Fig. 2, the proper motion μ has mean directions PA $= -50^\circ \pm 31^\circ$ and $-62^\circ \pm 11^\circ$ and magnitudes 0.10 ± 0.04 and 0.24 ± 0.04 m arc s yr⁻¹ during 1981 and 1982 respectively. The above estimates

indicate that there has been an apparent acceleration of the component. The proper motion of C_4 is less than that of C_3 and C_2 which had $|\mu| = 0.31$ and $0.42 \text{ m arc s yr}^{-1}$ respectively in 1982. This difference and the above acceleration could be due to actual differences/changes in the bulk velocity or in the angle between the velocity vector and the line of sight. With $k = H_0/55$ the apparent transverse velocity of C_4 in a co-moving frame was $(9.9 \pm 1.6)k^{-1}c$ during 1982.

Cohen *et al.*⁵ recently reported evidence that (1) during 1972–82 component C_2 moved radially at PA $\sim -75^\circ$ from $r = 1$ to 4 m arc s, and (2) C_3 at similar radii had a different PA -89° . Their conclusion is based on high-quality maps from 1979 to 1982, and less reliable data from 1971 to 1976. This result appears to be contradicted by the direct evidence shown above, that the track is not radial. We see three possible explanations for the discrepancy: (1) a component may have different behaviour at $r < 0.6$ and $> 1.0 \text{ m arc s}$. (2) Different components may follow different paths due to either precession or dynamical effects which move the jet. (3) The interpretation of the 1971 to 1976 data may be incorrect. Re-analysis (ref. 13, and J.A.B. *et al.*, in preparation) of the 1974.5 data of Wittels *et al.*¹⁴ show that this possibility is unlikely.

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Superluminal acceleration in 3C345

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The superluminal quasar 3C345 has a curved, one-sided jet-like radio structure^{1,2}. Ejected material has been observed travelling at apparent speeds of 13–17c (ref. 3). We report here new observations at 22 GHz which show that the most recently ejected component⁴ is not moving radially away from the compact radio core, but along a trajectory which could be interpreted as either a curved path originating in the compact core, or a straight line, in which case the origin of ejection is not coincident with the compact radio core. The observations provide evidence of acceleration of this component.

The quasar 3C345 ($z = 0.594$) was observed with a VLBI network at a frequency of 22 GHz at three different epochs—2 April 1981 (1981.25), 3 June 1982 (1982.42) and 2 February 1983 (1983.09). The observations are described in detail elsewhere (ref. 4, and L.B. *et al.* and J. Biretta *et al.*, in preparation). The observed structure at the first and third epochs is shown in Fig. 1; the structure from the second epoch is given elsewhere (L.B. *et al.*, in preparation). Earlier observations of 3C345 at 22 GHz show an extension in a similar position angle to that seen in the first epoch described here⁵, but we have not included

these observations in the present discussion as they did not include closure phases and fewer telescopes were involved, thus the quality is not comparable with that of the present observations.

The position of the western component relative to the eastern one at the second epoch is shown by the crosses in Fig. 1. The projected positions of the western component relative to the eastern component are given in Table 1. The errors given in Table 1 were estimated as follows: for each epoch a model consisting of two components with gaussian brightness distributions was assumed. The separation, r , of the two components was fixed and the other parameters were allowed to vary until the best fit was obtained to the closure phases. This was done for a range of r values which bracketed the optimum value. At the optimum separation, a χ^2 of 1.9 and 1.5 per degree of freedom was obtained for epochs 1 and 3, respectively. The limits on r given in Table 1 are those within which the fit is degraded by a factor 1.5 in χ^2 relative to the optimum value.

The same procedure was followed to determine the uncertainty in the position angle, θ , by setting this to certain values, and allowing the other parameters, including r , to vary. The errors for the second epoch have been interpolated from those of epochs 1 and 3. This is justified because of the similarity of the observations at all three epochs. The degradation of the fits appear as systematic deviations from the data. If the assumed models were actually correct these systematic deviations would be highly significant. However, it is clear that the real brightness distributions are more complex than assumed in our model and therefore that the formal χ^2 per d.f. cannot be directly converted into a confidence level. The value of applying this time-consuming method to determine uncertainties of the parameters of the emission regions is that it is a well defined quantitative procedure which is preferable to the subjective estimates usually applied in this type of model fitting. Based on our experience with model fitting and the maps of the second and third epochs, we believe that the resulting errors are roughly 5σ for the positions of the west component relative to the east component.

We will assume in this discussion that the eastern component is the radio core, that is, the flat spectrum component nearest the centre of activity; justification for this assumption is given elsewhere (J. Biretta *et al.*, in preparation). Following the convention suggested by Unwin³ we will refer to the core as component D and the western component as C_4 . We extend this convention by adding subscripts to designate epoch of observation: thus, C_{41} , C_{42} and C_{43} refer to epochs 1, 2 and 3, respectively.

We now turn to the apparent velocity. We assume throughout that $q_0 = 0.05$ and $H_0 = 55 \text{ km s}^{-1} \text{ Mpc}^{-1}$. From the relative positions of C_{41} , C_{42} and C_{43} in Table 1, we calculate an apparent velocity between the first and second epoch and the second and third epoch. These are shown in Table 1, together with values of $\beta_{\text{app}} = v_{\text{app}}/c$. Note that the apparent velocity (v_{app}) increases from $6.8c$ to $11.2c$ and that the difference is significant at about the 5σ level. There is thus clear evidence of acceleration in component C_4 . Observations of 3C345 at 10 GHz have been reported in the accompanying paper⁶. These observations are in good agreement with ours, and provide independent evidence of the acceleration and non-radial motion of C_4 relative to D.

We now consider two possibilities: (1) component C_4 did not emerge from the core, or (2) component C_4 emerged from the core, and is travelling along a curved path. It is not possible to distinguish between these two alternatives on the basis of the present observations. The position of C_4 relative to D for the three epochs is plotted in Fig. 2 with 3σ error bars. The observed trajectory is roughly rectilinear beyond 0.3 m arc s from the core. Therefore, observations are required at even higher frequencies and at earlier epochs after the appearance of new components to determine whether or not the trajectories pass through D.

The superluminal expansion could be produced by reflection on a screen, but deceleration is more likely to occur in screen models than acceleration⁷, and we do not consider this possibility

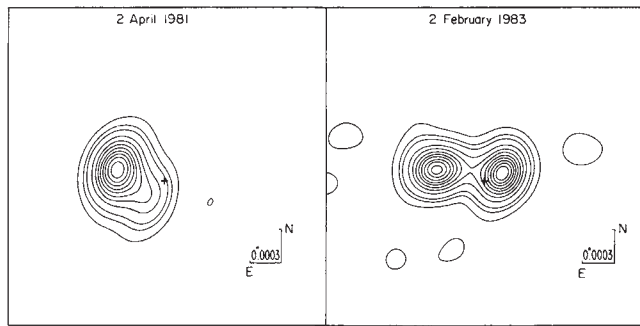


Fig. 1 Hybrid maps of 3C345 at 22 GHz: left, epoch 1 (2 April 1981); right, epoch 3 (2 February 1983). The crosses mark the position of the western component relative to the eastern component at epoch 2 (3 June 1982). Contour levels: -2, 2, 6, 10, 20, ... 90% of peak.

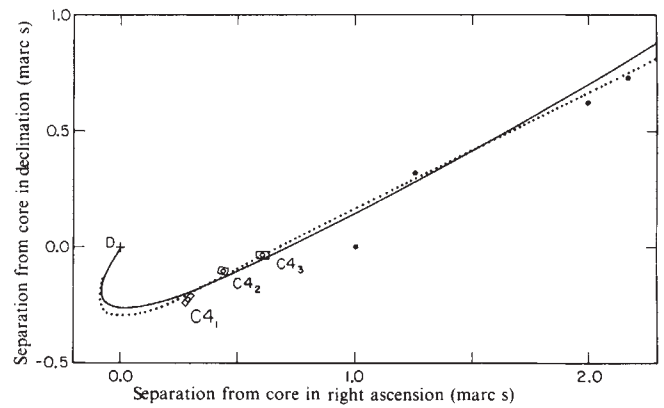


Fig. 2 The position of the new component C4 at three different epochs (open circles) relative to component D. The error boxes indicate roughly 3σ errors (see text). The solid line is a hyperbola chosen to fit both the inner (○) and outer (●) points. The dotted line is straight beyond 3 marc s and provides a slightly better fit to the observations.

Table 1 Parameters of superluminal motion in 3C345

Epoch	Component	Observed values		Derived values			
		r (marc s)	θ	Proper motion (marc s yr ⁻¹)	β apparent*	ϕ	β
	D	0	—			1.8°	
1981.25	C4 ₁	0.37 ± 0.02	$-128^\circ \pm 8^\circ$	0.17 ± 0.05	6.8 ± 2.0	2.2°	0.995 ± 0.0015
1982.42	C4 ₂	0.45 ± 0.035	$-103^\circ \pm 3^\circ$			2.7°	
1983.09	C4 ₃	0.61 ± 0.04	$-93^\circ \pm 2.5^\circ$	0.27 ± 0.10	11.2 ± 3.9	3.0°	0.997 ± 0.0010

Errors are $\pm 5\sigma$.

* Values of $H_0 = 55 \text{ km s}^{-1} \text{ Mpc}^{-1}$ and $q_0 = 0.05$ have been assumed.

further here. For the remainder of this discussion we will assume that superluminal motion is caused by relativistic motion of the emitting material towards the observer.

If the motion of C4 is rectilinear (for example, case 1) the trajectory has an impact parameter with the core of 0.3 marc s, that is, 2.4 pc, which is much larger than the size of the central engine. Such a situation could occur if there were more than one supermassive body in the nucleus of 3C345 (ref. 8). It appears somewhat contrived because of the near equality of flux densities of D and C4 which are presumably both strongly relativistically beamed, but it certainly cannot be excluded at this stage.

We now consider the second alternative, that is, we assume that component C4 emerged from component D and is travelling at a small angle, ϕ , to the line of sight along a very slightly curved trajectory. In this model one would expect the path to be curved towards D, whereas it appears to be slightly curved away from D. However, if this curvature is real, it could be due to small oscillations in the trajectory which would be amplified by projection effects, and it therefore does not contradict the model. The approximately straight path of component C4 and the offset of this path from the core place interesting limits on the trajectory of the component on this model. Present values of the Lorentz factor, γ , suggest an upper limit of $\phi \leq 5^\circ$. It seems likely that the bending force responsible for the intrinsic curvature of the jet decreases with distance from the core. We assume, therefore, that the path is hyperbolic. The angle through which the jet bends between D and C4, $\Delta\phi$, must be $< 1/\gamma$ but this small intrinsic curvature is greatly amplified by projection. An attempt to fit the present observations and the positions of some more distant components⁴ with a hyperbolic curve is shown by the solid line in Fig. 2. In this particular example the angle between the line of sight and the plane of the hyperbola is 0.7° . The corresponding angles, ϕ , are given in Table 1. The previous component ejected from 3C345, C3, is following a path at

position angle $\sim -90^\circ$, that is, significantly different from the path shown in Fig. 2 (J. Biretta *et al.*, in preparation). Thus, we are not suggesting that all components in 3C345 follow this path identically. We include it here because it connects the trajectory of C4 with the trajectories of some older components. This curve is not a very good fit to the observations and clearly a better fit can be obtained by assuming that the path departs from a hyperbola and is rectilinear beyond C4₁; such a path is shown by the dotted line in Fig. 2. A discussion of the cause of the bending is beyond the scope of this letter but will be given elsewhere (J. Biretta *et al.*, in preparation).

The apparent velocity is given by $v_{\text{app}} = v \sin \phi / (1 - v/c \cos \phi)$, which has a minimum for $\sin \phi = 1/\gamma$. Thus, it is possible that the speed of C4 along the jet is constant, and that the observed acceleration is due solely to a change in ϕ . However, for a simple hyperbolic path such as that in Fig. 2, the change in ϕ is too small to account for the observed acceleration (see Table 1), and an increase in the speed along the jet is required. Of course, the acceleration could be caused simply by changes in ϕ for more complex planar or non-planar paths.

Previous observations provide some evidence for acceleration in ejecta as they move away from the core³, however this is the first time that it has been seen in a single component. If acceleration occurs in all components ejected in 3C345 this would enable us to reconcile the low apparent velocities deduced in this object in the early 1970s from model fitting, at small separations, with the high values deduced, at high separations, from later hybrid maps³.

The two alternative interpretations of the trajectory of C4 considered above are kinematically different, but they are not necessarily physically different: for example, R. D. Blandford (personal communication) has suggested that the ejection cone from the core might be quite broad, and that the observed components might be due to shocks or plasma instabilities within

such a broad cone. (A broad cone of ejection has been invoked previously^{4,9} to explain features in 3C273 and in NGC 1275.) The apparent trajectory of C4 would then be due both to motion of these shocks or instabilities across the ejection cone and to the outward flow along the jet. Clearly, such a situation could give rise to a variety of trajectories, many of which do not intersect the core but all of which have the same physical origin.

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Lake of Lugano and topographic waves

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During the summer of 1979, wind, water temperature and current measurements were made in the Lake of Lugano, Switzerland, to study its response to meteorological forcing. Analysis of the collected data revealed the presence of wind-induced internal gravity wave seiches with periods of 24 h and less¹. However, during this study, an intriguing 3-day oscillation in the temperature and horizontal current records was also noted¹. We present here evidence which suggests that this low-frequency oscillation is a free topographic Rossby wave that travels around the lake in an anticlockwise direction.

Topographic waves (also known as vortex modes, rotational modes and second-class waves) can only exist in a rotating fluid with bottom topography. In the Northern Hemisphere, where the Coriolis parameter is positive, shallower water is always to the right of the direction of phase propagation². The current motions associated with topographic waves are obtained from the solution of a vorticity equation, which states that the time rate of change of relative fluid vorticity is produced by the stretching and contracting of water columns moving across the depth contours. In the ocean such waves have been frequently observed on the shelf³, the continental rise⁴ and the Charlie Gibbs Fracture Zone⁵. They have also been detected propagating anticlockwise around some of the Laurentian Great Lakes^{6,7}, which are of large enough horizontal scale for rotational (Coriolis) effects to be important⁸. Because the Lake of Lugano is so small (about 1.5 km wide and 17 km long), one does not expect rotational effects to have a dynamical role. Indeed, many investigators⁹ would argue that a 3-day oscillation in such a small lake must be due to direct wind forcing. However, a cross-spectral analysis of the wind and temperature fluctuations from three lake moorings indicates that this is not the case.

Figure 1b shows, for the southern end of the lake, the power spectra of low-passed (30-h cutoff) longshore wind fluctuations and fluctuations of the mean temperature displacement function. The latter quantity is representative of the mean isotherm–depth time series, which itself is obtained from thermistor chain records covering the depth range 37–57 m below the lake surface¹. The sharp peak at 80 h in the Ferrera temperature spectrum marks the aforementioned 3-day oscillation. This signal is clearly not

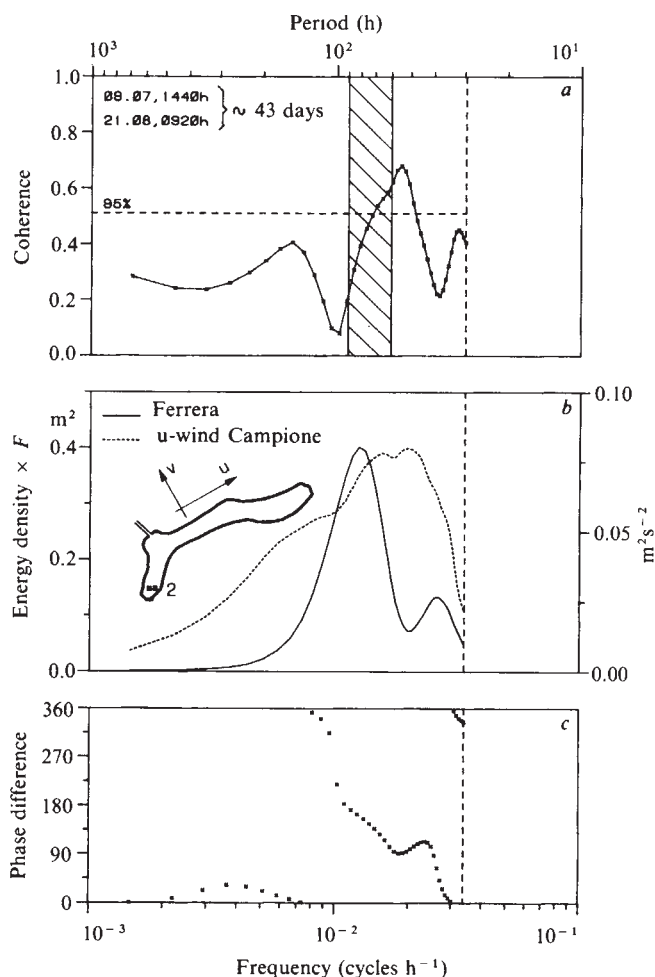


Fig. 1 Variance spectra (energy density times frequency, b), coherence (a) and phase (c) of the low-passed *u*-component of Campione wind (measured 3 m above water surface) and Ferrera mean temperature displacement function. The vertical dashed lines mark the 30 h cutoff, and the shaded strip represents the 60–90 h period band. The locations of Campione and Ferrera are marked by the squares labelled 2 in the inset. Right and left variance scales apply to the wind and temperature displacement respectively. The phase difference is defined so that the phase of the Ferrera data (whose variance is shown as a solid curve) leads that of the Campione data (whose variance is shown as a dotted curve).

simply due to direct wind forcing as the wind spectrum shows a broad peak at a shorter period and the coherence at 80 h (Fig. 1a) between the two sets of fluctuations is just below the 95% confidence level. Also, the phase difference around 80 h (Fig. 1c) does not have the 180° shift associated with a resonant response. Spectra of the longshore wind at both central and northern lake stations have very little energy in the 60–90 h band, and at both locations the coherence between wind and temperature in this band is very low¹⁰. However, the temperature spectra have well-defined peaks near the 3-day period (see Fig. 3). These results strongly suggest that the 3-day signal represents some type of natural oscillation of the Lake of Lugano which, however, is probably excited by wind gusts every 2–3 days at the southern end of the lake.

A fairly regular 74-h oscillation throughout the lake can be clearly seen in the time series of the mean temperature displacement functions for five lake locations (see Fig. 2b). Approximately six cycles can be identified during the period 12–30 July, and following a storm event on 31 July–1 August, another five cycles could be identified in the continuation of these time series¹⁰. Examination of the wind records (Fig. 2a) clearly shows no regular 74-h oscillation, but rather a pronounced spatial inhomogeneity in the wind field, with 2–3 day fluctuations being the largest at Campione (2). As suggested by the relatively high

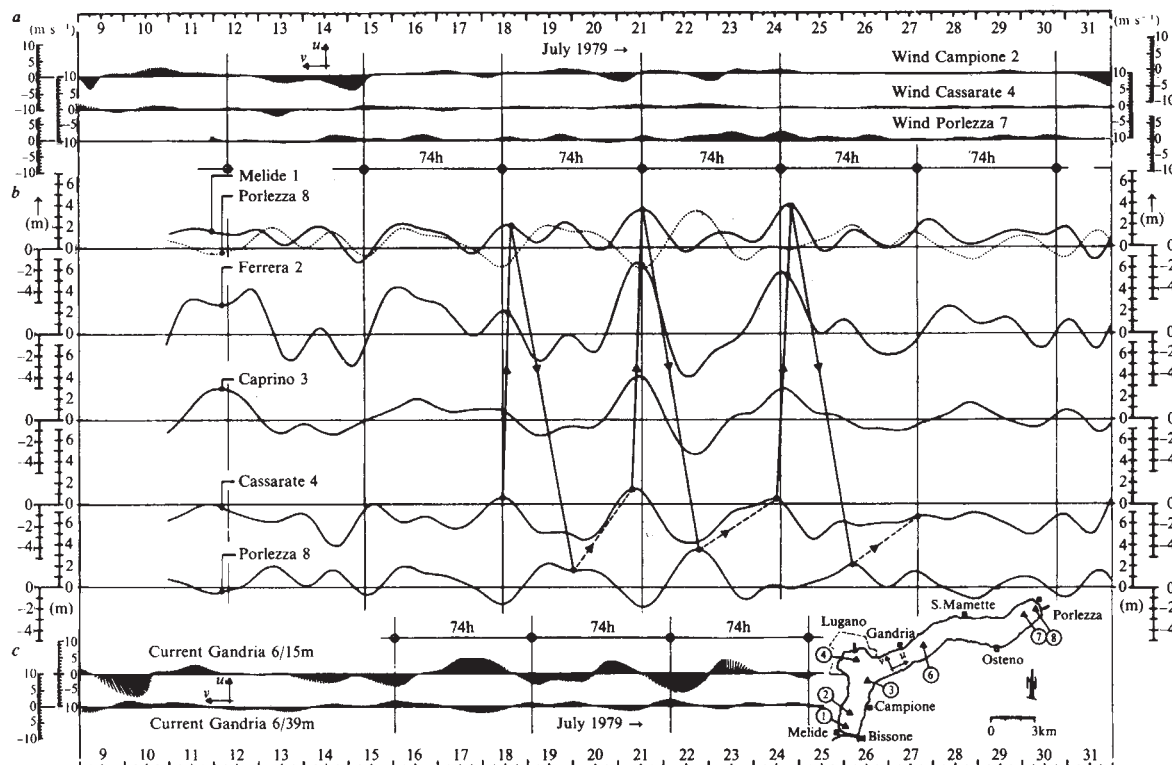


Fig. 2 Time series for the Lake of Lugano measurements from 9 to 31 July 1979. The data were smoothed with a rectangular filter to eliminate all processes with periods shorter than 30 h. *a*, Stick plots of the winds at stations Campione (Ferrera) (2), Cassarate (4) and Porlezza (7) (see inset for locations). *b*, The mean temperature displacement functions at Melide (1), Ferrera (2), Caprino (3), Cassarate (4) and Porlezza (8). *c*, Stick plots of the currents at 15- and 39-m depths at station Gandria (6). For all wind and current plots, u and v have the orientation shown in map. Vertical lines mark a period of 74 h, and the slanted lines indicate the progression of the wave crests (marked with dots). The slope of the lines connecting Melide and Porlezza wave crests is greater in magnitude than the Cassarate-Melide slope because of the larger separation between Melide and Porlezza. The different slopes thus do not imply different phase speeds. Similar remarks apply to the even larger slope of the lines between Porlezza and Cassarate wave crests.

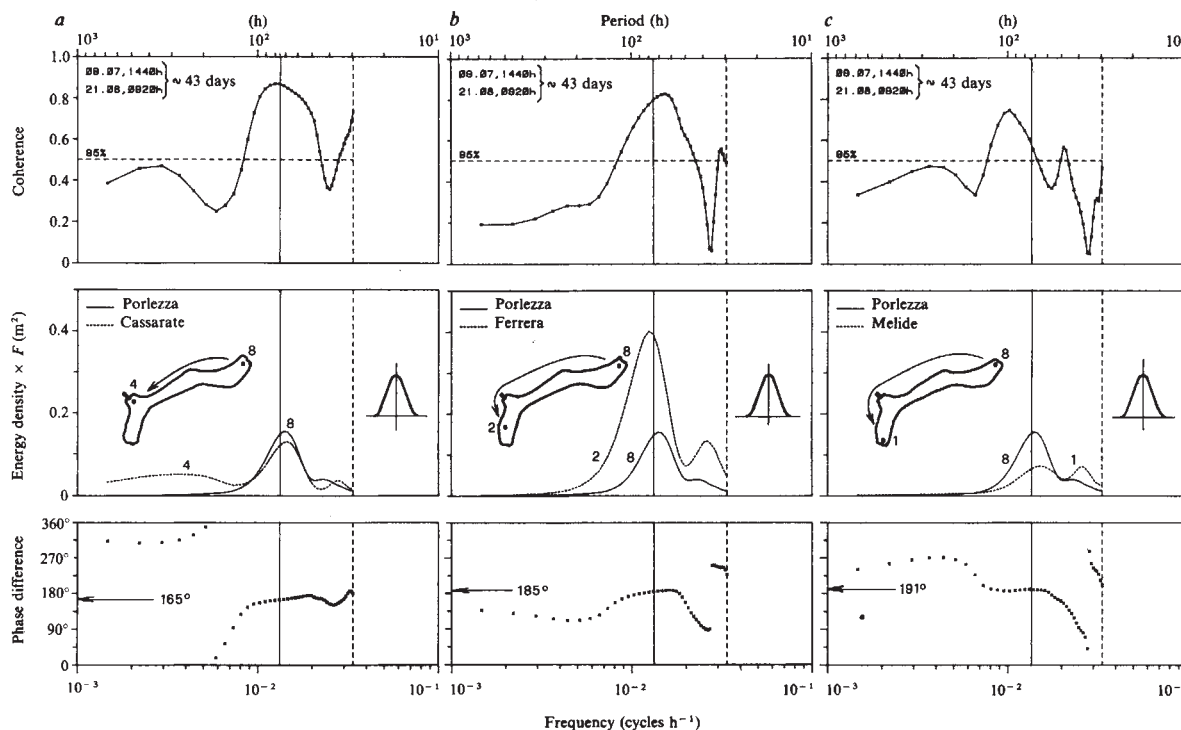


Fig. 3 Variance spectra, coherence and phase of three low-passed time series pairs of the mean temperature displacement function: *a*, Porlezza (8)/Cassarate (4); *b*, Porlezza (8)/Ferrera (2); *c*, Porlezza (8)/Melide (1). Insets show the location of each station pair, the arrow indicating the direction of phase propagation. Windows in the variance plots indicate the tapering of the spectra. The vertical solid line marks the period of 74 h, and the arrow on the left of each phase diagram indicates the phase difference between the time series pair at that period.

wind-temperature coherence at 80 h (Fig. 1a), the Campione (2) winds probably excite the 3-day lake oscillations. In addition to the temperature oscillations seen in Fig. 2, three cycles with a 74-h period can be observed in the current records (Fig. 2c). The 180° phase shift with depth, the surface intensification and the 0.75-day phase shift from the temperature time series are features consistent with a theoretical two-layer model for topographic waves in an elliptical basin¹⁰. We now focus on the observed temperature phase difference between different pairs of stations, and show that these relations are also consistent with an anticlockwise travelling topographic wave.

Note that the temperature displacement time series for the four southwestern stations (1)–(4) generally shows a high when that at Porlezza (8) at the other end of the lake has a low, and vice versa. In addition, the slanted lines in Fig. 2b show a propagation of wave crests in the order Cassarate, Ferrera, Melide, Porlezza and then back to Cassarate. This progression suggests that there is a free wave with a 74-h period that travels anticlockwise around the lake. For an elliptical basin model of the Lake of Lugano, the gravest-mode topographic wave has a period of 75.6 h and travels anticlockwise around the lake¹¹, in agreement with the observed progression at a similar period (74 h). Also, the wave-induced interfacial displacements at the ends of the theoretical model are 180° out-of-phase¹⁰, a feature

which is also generally consistent with the data in Fig. 2 (see the Melide time series, with the dotted Porlezza time series superimposed).

To statistically verify the anticlockwise progression of the 74-h signal, the variance spectra, coherence and phase of the mean temperature displacement functions for the station pairs 8/4, 8/2 and 8/1 were computed. Figure 3 shows that the variance spectra have maxima between 70 and 80 h; and in this band the coherence between the time series pairs is very high. The mean period of the variance maxima is 74.5 h. If this is the period of a topographic wave travelling around the lake, then the phase difference for the station pairs 8/4, 8/2 and 8/1 at 74 h should increase in this order, ranging from about 160° to 180°. The computed phase differences at 74 h are 165°, 185° and 191° (Fig. 3), which is consistent with the wave theory. The phase difference 8/3 was also computed¹⁰, but the value (182°) is smaller than the theoretical prediction (about 200°). The reason for this discrepancy is unknown, but may be related to the interference of the nearby Cassarate (4) fluctuations on the Caprino (3) record. The phase differences for the station pairs 4/2, 4/1, 4/3 and 4/8 were also computed¹⁰, and the results are entirely consistent with the above phase calculations which used Porlezza (8) as a reference.

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Reconstruction of eastern Gondwanaland

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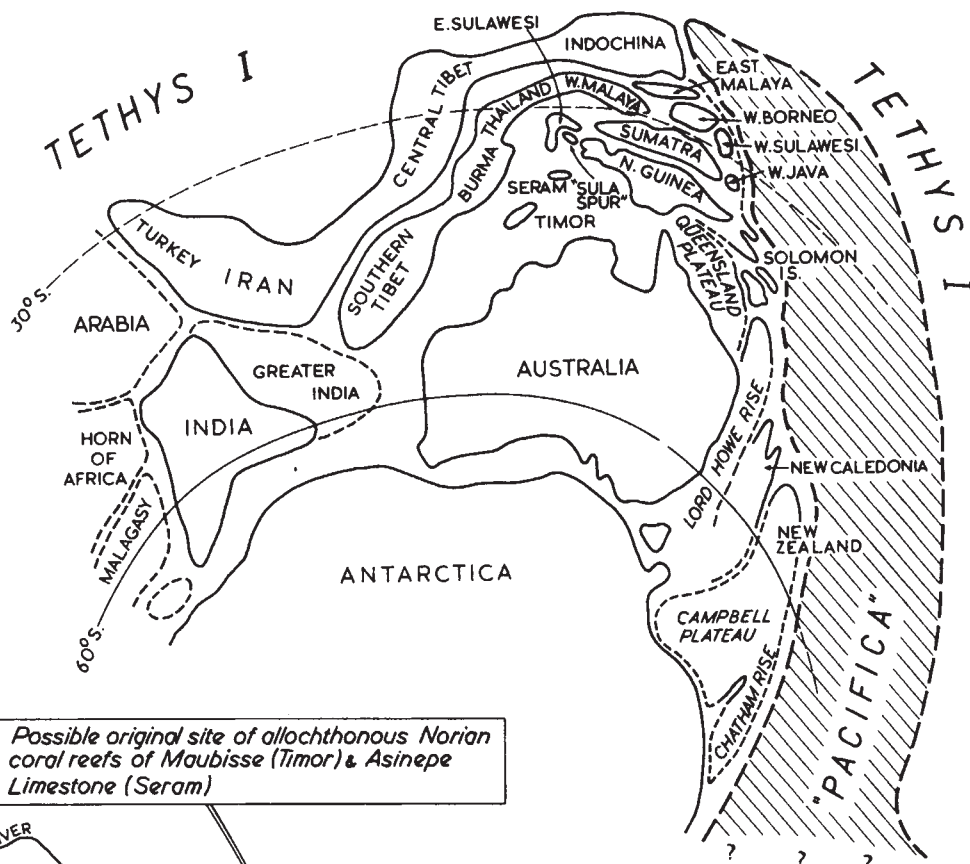
Substantial additions of large continental blocks to the Smith *et al.*¹ model of eastern Gondwanaland are proposed here largely on the basis of the distribution of key land floras, tropical and subtropical marine faunas, lithofacies patterns and the identification of a Triassic magmatic arc that characterized all the eastern margin of Gondwanaland. The continental fragments that must have been rifted from northern Australia–New Guinea in the Jurassic are identified as south Tibet–Burma–Thailand–Malaya and Sumatra. The original site of deposition of the subtropical Permian limestones and tropical late Triassic limestones, overthrust onto the northern margin of Australia by the late Cenozoic collision², is located in this greater Gondwanaland. Palaeomagnetic data, except those from Malaya³, support this reconstruction.

The outstanding problem in reconstructing eastern Gondwanaland has been the difficulty in identifying the continental blocks that in the Jurassic must have been rifted from northern Australia and central New Guinea. Hamilton⁴ suggested Indochina, the Malay Peninsula and Sumatra were rifted in the Jurassic from the continental margin of, what is now, central New Guinea. Mitchell⁵ proposed that Burma and the Thai–Malay Peninsula lay adjacent to northern Australia during the Palaeozoic, separating from Gondwanaland in the Permo-Triassic. He argued for the continuity of southern Tibet with this Burma–Thai–Malay block. Ridd⁶ and Tarling⁷ suggested that South-East Asia formed part of Gondwanaland during the late Palaeozoic.

We propose here a qualitative reconstruction of eastern Gondwanaland (Fig. 1) based on the presence of late Palaeozoic glacial deposits, the distribution of key land floras, tropical and subtropical marine faunas and facies that can be related to the margins of Australia and New Guinea and to parts of South-East Asia. This reconstruction takes account of the limited available palaeomagnetic data for South-East Asia. An important line of evidence is the identification of a major suite of calc-alkaline and acid volcanics and granite intrusions of mid-late Triassic age that unites eastern Australia, central New Guinea, Sumatra, Borneo, Malaya, Thailand, Burma and south Tibet into an active continental margin during the late Permian to late Triassic. This is related to contemporaneous Tethys II ocean spreading associated with the dispersal of Asian and possibly also 'Pacifica' fragments of Gondwanaland (Fig. 2). The palaeogeographical origin of the allochthonous terranes in western North America is uncertain^{8,9}. The palaeomagnetic and faunal evidence of the Wrangellia terranes¹⁰ seems to me on balance to favour their derivation from eastern Gondwanaland by Permo-Triassic ocean spreading⁸ but that interpretation remains speculative awaiting further study. It is adopted here partly because it offers an attractive explanation for the evolution of a magmatic arc in eastern Australia–New Guinea–Sumatra, western Borneo and Malaya.

The core of this reconstruction (Fig. 1) is the Antarctic–India–Australia assembly of Smith *et al.*¹ that appears in all three configurations of Pangea discussed recently by Hallam¹¹. The reconstruction of the eastern margin of Australia follows Griffiths¹². The 60° S, 30° S and 0° parallels have been plotted from Smith *et al.*¹. All the remaining areas have been plotted on the assessment of their geological affinity with some modest support from palaeomagnetic data which are very scarce for pre-Cretaceous rocks of South-East Asia^{13,14}. Most of the limited palaeomagnetic data support these reconstructions (Figs 1, 2). For example, the evidence that during the early Mesozoic Sumatra appears to have been situated about 10°–20° south of its present latitude and during the late Mesozoic drifted north

Fig. 1 Qualitative reconstruction of eastern Gondwanaland during the mid-Carboniferous (320 Myr). Reconstruction of the Banda Arc region of the northern Australian margin (Timor, Seram, eastern Sulawesi) follows an earlier proposal³⁷. The evidence for the other elements is discussed in the text. Present coast lines or outlines are for reference only.



Possible original site of allochthonous Norian coral reefs of Maubisse (Timor) & Asinepe Limestone (Seram)

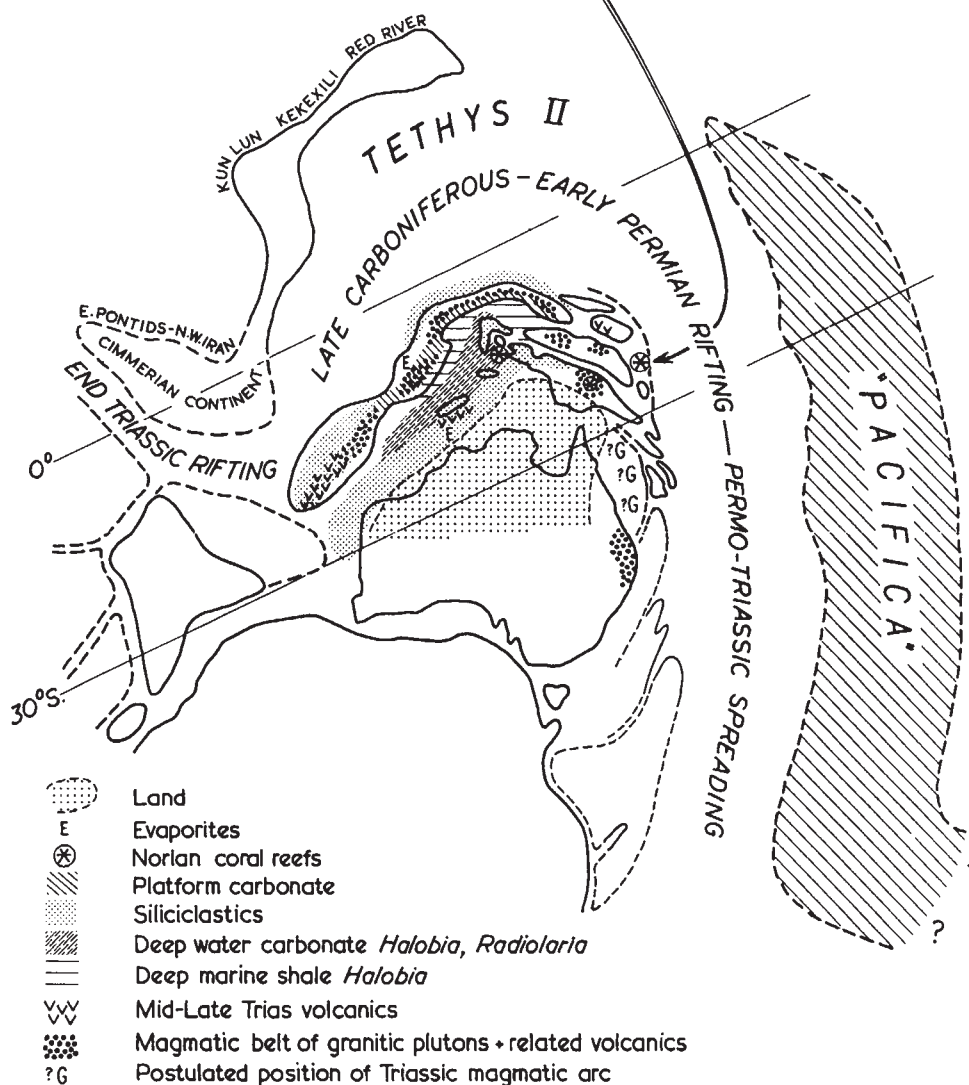


Fig. 2 Qualitative reconstruction of eastern Gondwanaland during the late Triassic (220 Myr) showing some of the data on which the identification of the fragments rifted from the northern Australian-central New Guinea margin has been based. Attention is drawn to the magmatic arc forming the continental margin of eastern Gondwanaland which appears to be related to the spreading of Tethys II ocean. The Kun Lun-Kekexili-Red River suture marks a late Triassic tectonic collision zone with Asia^{5,19,40}. The east Pontids and north-west Iran formed a possibly younger collision suture¹⁷. 'Pacifica' included continental fragments, oceanic plateaus and perhaps island arcs⁵. The position of Pacifica and the Iran-Central Tibet-Indochina block and the amount of spreading of Tethys II is schematic.

accompanied by a 30° clockwise rotation¹³ fits the configuration shown in Figs 1 and 2. Sasajima *et al.*¹⁵ suggested on the basis of its polar path that Sumatra was part of Gondwanaland. On the other hand, the Malay Peninsula and Indochina were said by Haile¹⁴, on the basis of palaeomagnetic data, to be unlikely to have formed part of Gondwanaland, at least since the late Palaeozoic³. However, as these Malay and Indochinese rocks have suffered severe deformation and metamorphism, I have allowed the strong geological indications for Malaya and Indochina having been part of Gondwanaland in the late Palaeozoic to have influenced this reconstruction.

The case for part of Turkey, Iran and Central Tibet having been derived from northern Gondwanaland was discussed by Ziegler *et al.*¹⁶ and Sengör *et al.*¹⁷ who referred to these rifted fragments as the Cimmerian continent. McTavish¹⁸, on the basis of Triassic conodont distributions argued for the presence of Iran adjacent to Arabia and Malaya adjacent to northwestern Australia and Timor as part of the Gondwanaland margin. The presence in central Tibet of Carboniferous tillites^{19,20} suggests it formed part of Gondwanaland in the late Palaeozoic. The similar Cathaysian land flora²¹ of central Tibet, Indochina, Thai-Malay Peninsula with links into New Guinea and Anatolian Turkey strengthen the case for this reconstruction (Fig. 1). This is also supported by the distribution of the Verbeekiniidae²², a group of Permian fusulinid foraminifera linking Turkey, Iran, Central and southern Tibet, Indochina, Burma, Thailand, Malaya and Sumatra. Some other larger Permian fusulinids are also found in these areas, in Borneo²³ and the allochthonous Permian Maubisse Limestone of Timor²⁴ thrust onto the Australian margin from the north in the Pliocene².

The distribution of a distinctive Permian brachiopod fauna in Thailand, New Guinea, the allochthonous Maubisse limestones of Timor and in western Australia²⁵ provides a vital link of these subtropical faunas with the northern margin of eastern Gondwanaland. The distribution of shallow marine limestones of Permian and late Carboniferous age with waagenophylliid corals, oldhaminid brachiopods, calcareous algae and a high diversity of other invertebrate forms in south Tibet¹⁹, Burma^{5,26}, Thailand^{6,27,28}, Malaysia^{27,28} and Sumatra^{4,28,29} provide support for grouping these areas at the northern margin of eastern Gondwanaland^{6,7}. Stauffer and Gobbett²⁷ and Stauffer²⁸ argued that these faunas and lithofacies indicate that South-East Asia was located in the tropics or subtropics during the late Palaeozoic and therefore this was evidence against South-East Asia having been part of Gondwanaland. However, Figs 1 and 2 offer an explanation for overcoming their objection, and using their argument as evidence for this configuration especially bearing in mind the distribution of the Cathaysian²¹ land floras of Tibet and South-East Asia and mixed Gondwana-Cathaysian land floras of New Guinea, Thailand and Turkey^{21,28}.

The evidence for a late Triassic collision with Asia along the Kunlun-Kekexili-Red River zone (Fig. 2)^{5,19} accords with central Tibet-Indochina having been rifted from Gondwanaland with the spreading of a new ocean Tethys II during the Permo-Triassic. This has considerable palaeogeographical importance as it can account for the evolution of the remarkable Triassic magmatic arc in south Tibet¹⁹, Burma²⁶, Thai-Malay Peninsula⁵, Sumatra^{4,29}, Borneo²³, the Kubor range of New Guinea³⁰ and eastern Australia³¹.

Another feature of this new reconstruction is the explanation it offers for the original site of deposition of the fossiliferous Permian Maubisse limestones³² that are found as thrust sheets that moved southwards onto Timor in the Pliocene². These limestones contain large fusulinids with rich brachiopod, coral, crinoid, blastoid, ammonoid faunas of subtropical aspect²⁵. They are closely associated with late Triassic limestones containing colonial reef corals³³. Similar Triassic colonial coral reefs are also found in thrust sheets forming the Asinepe limestone in Seram². These late Triassic (Norian) coral reef limestones in

the allochthon of Timor³³ and Seram^{2,34} appear to be identical in age with autochthonous coral reef limestones in Misool³⁵ and the Kubor range of central New Guinea³⁰.

Carter *et al.*³⁶ argued that these allochthonous Permo-Triassic limestones were thrust during the Pliocene collision onto the Australian margin from their pre-Pliocene position in the fore-arc basement of the Banda volcanic arc. This led to the suggestion³⁷ that the original site of deposition of these allochthonous Permo-Triassic limestones was the eastern Sundaland region of south-east Borneo and south-west Sulawesi. Chamalaun^{38,39} argued from palaeomagnetic evidence that the Maubisse Limestone of Timor appears to have a similar Permian palaeomagnetic pole to that of Australia, and could not be separated from the para-autochthonous Permian Cribas Formation of Timor. The interpretation proposed (Fig. 2) removes the apparent contradiction between the local geology and the palaeomagnetic data.

Another important aspect of these late Triassic reef corals is the support they provide for the late Triassic northward excursion of Australian Gondwanaland proposed by Smith *et al.*¹. They show New Guinea making a brief visit into tropical latitudes during the late Triassic before returning south to a colder position. That brief tropical visit is marked by the deposition of coral reef limestones in Misool³⁵, central New Guinea³⁰ and as interpreted here in eastern Sundaland³⁷.

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Sulphur isotopic compositions of deep-sea hydrothermal vent animals

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Dense animal assemblages consisting of vestimentiferan worms, brachyuran crabs and giant clams have been found clustered around deep-sea hydrothermal vents in unusual ecosystems that appear to be independent of photosynthetically produced nutrients. Reduced compounds such as H_2S , H_2 and CH_4 are spewed from the vents into the cold, oxygenated bottom water^{1,28}, and it has been suggested that chemoautotrophic bacteria are important primary producers of organic nutrients utilized by the vent fauna²⁻⁷. In some instances, sulphur-oxidizing bacteria may perform this function through growth as symbionts^{8,9} within tissues of host animals (clams and vestimentiferans). To gain further insight into sulphur metabolism in these unusual food webs, we have analysed the stable sulphur isotope ratios ($^{34}S/^{32}S$) of Pacific vent fauna and find $\delta^{34}S$ values close to 0‰. These values approximate the +1 to +4‰ range observed for sulphur-bearing minerals at the vents, and indicate that this specialized fauna utilizes sulphur derived from the vents (rather than from seawater sulphate) during growth and metabolism.

Dried (60 °C) samples of vestimentiferan (*Riftia pachyptila* Jones) and clam (*Calypotgena magnifica* Boss and Turner) tissues were obtained from Scripps Oceanographic Institution; muscle was dissected from dried crab (*Bythograea thermydron*) claws obtained from the University of California, Santa Barbara. White *Riftia* tubes were freed of small black particles by rinsing; only clean tissue was analysed. All samples were ground to fine powder with mortar and pestle, then suspended in 10 volumes of 0.1 M LiCl and successively shaken twice vigorously at 37 °C for 30 min to remove seawater sulphate¹⁰. The supernatant fluid was removed after centrifugation and the pellets dried at 60 °C. Samples were combusted under 30 atm O_2 in a Parr bomb and sulphur recovered as $BaSO_4$. Sulphur dioxide was generated from 5–30 mg samples of $BaSO_4$ by thermal decomposition¹¹, and analysed on a Nuclide 6-60 isotope ratio mass spectrometer. Results are expressed in conventional $\delta^{34}S$ notation relative to sulphur in Canyon Diablo troilite¹² where

$$\delta^{34}S = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 10^3 \quad \text{and} \quad R = {}^{34}S/{}^{32}S$$

The sulphur isotopic composition of the hydrothermal vent faunas differs significantly from that of previously studied oceanic faunas (Fig. 1). In contrast to the -5 to +5 values of the vent fauna, values for animals from marine food webs based on phytoplankton have sulphur isotopic compositions much nearer that of seawater sulphate (+20‰), the usual source of algal sulphur (Fig. 1). The +13 to +20‰ values in marine animals from algal-based food webs are consistent with observations that (1) sulphur in marine algae usually has $\delta^{34}S$ values in the +15 to +20‰ range^{13,14}, and (2) animals cannot reduce sulphate for protein synthesis and therefore depend on other dietary sulphur sources¹⁵. The lower isotopic values observed in the vent fauna (Fig. 1) suggest that these animals do not obtain their sulphur from marine algae. Rather, the isotopic similarity of sulphur in vent animals to sulphide minerals at the vents (Fig. 1) suggests that vent sulphur is the major source of sulphur for vent animals.

Bacteria presumably are intermediates in the transfer of vent sulphur through the food web to the vent fauna. Whereas animals can incorporate minor amounts of reduced inorganic

Table 1 Isotopic compositions of sulphur sources and of sulphur in hydrothermal vent fauna

Sample	$\delta^{34}S_{\text{CDT}}$ (‰)*
Seawater sulphate, Gulf of Mexico	+20.1, +20.1
Sulphide minerals from vent†	+1.3 to +4.1
<i>Riftia pachyptila</i> ‡	
Tubes	A§ +4.7
B	0, +0.7
Vestimentum tissues	C -2.1
D	-4.7
Trophosomal tissues	C -1.5, -2.0
D	-3.8
Clams, <i>Calypotgena magnifica</i> ‡	
Foot	A +0.4
B	-1.7
Gill	A +1.5
Crabs, <i>Bythograea thermydron</i>	
Claw muscle	A -0.1, -0.4, -0.4
B	-0.7, -0.8

* Multiple entries indicate replicate analyses.

† 24 samples from 21° N site, see ref. 7.

‡ From 21° N site, see ref. 7.

§ Letters designate different individuals.

|| From Galapagos site, see ref. 1.

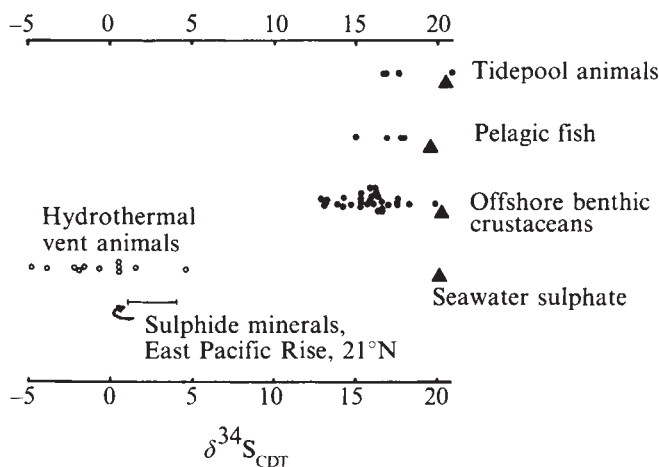


Fig. 1 $\delta^{34}S$ values of hydrothermal vent fauna compared with normal marine faunas. $\blacktriangle$, Seawater sulphate; $\circ$, $\bullet$, individual animal or tissue. Sources: tidepool animals, ref. 13; pelagic fish, ref. 14; offshore benthic crustaceans, ref. 26; seawater sulphate, collected in the Gulf of Mexico, this study; sulphide minerals, ref. 27.

sulphur compounds directly into cysteine^{16,17}, they usually oxidize the bulk of such sulphur compounds to sulphate¹⁸. Most commonly, animals obtain sulphur-containing amino acids from plants and microorganisms in their diets. Although we have not measured the isotopic composition of sulphur in the bacterial biomass (that is, food) at the vent, we expect isotopic fractionations associated with assimilative uptake of reduced or oxidized sulphur species to be small; accordingly, the bacteria would have vent-like $\delta^{34}S$ values. We base this opinion on observations that isotope effects during oxidation of sulphide and elemental sulphur are usually <3‰ (refs 19, 20), and that aerobic and anaerobic bacteria grown with sulphate have $\delta^{34}S$ values within 3‰ of their sulphur source^{21,22}.

The 9.4‰ scatter (Fig. 1, Table 1) among sulphur isotopic compositions of the vent fauna is interestingly large. In clam tissues and in *Riftia* trophosome and vestimentum tissues, sulphur is present as a mixture of organic sulphur and inorganic vent sulphides that are carried in the blood²³⁻²⁵. The crab muscle and secreted *Riftia* tubes contain only organic sulphur. The observed variation of isotopic abundances suggests that some

isotopic discrimination occurs during sulphur metabolism; binding and dissociation of sulphide from blood components or variations in bacterial oxidation pathways are possible mechanisms. Crabs are scavengers in this ecosystem, and their near-zero $\delta^{34}\text{S}$ values, close to the centre of the range observed in this work, are possibly representative $\delta^{34}\text{S}$ averages for the vent food web.

Oxidation of reduced sulphur compounds is apparently a major source of bacterial productivity at the deep-sea vents, and isotopic compositions presented here are consistent with a postulated dietary importance of sulphur-oxidizing bacteria^{8,9}. Future stable sulphur isotope analyses may also show a direct link between animals and reduced sulphur compounds in other less exotic environments—marine muds, sewage outfalls, meromictic lakes and so on—where these compounds commonly serve as important energy sources for bacterial productivity.

It is conceivable that vent fauna, in contrast to other animals, can use sulphide directly as a major precursor of organic sulphur compounds. On comparative biochemical grounds this seems unlikely, but requires further experimental study. Various observations, including ours, are consistent with the suggestion that primary productivity in vent ecosystems is due mainly to chemoautotrophic bacteria that can use reduced inorganic sulphur compounds as energy sources. Note, however, that other explanations are also possible. For example, primary productivity might be due in significant measure to bacteria that use H_2 or methane as an energy source, and these or other types of bacteria may convert sulphide to sulphur amino acids and so on, which are then used as biosynthetic sulphur sources by vent animals. In any event, the observed sulphur isotopic compositions clearly indicate that, in contrast to pelagic animals, vent fauna utilize an unusual local source of sulphur.

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New hominoid primates from the middle Miocene Chinji Formation, Potwar Plateau, Pakistan

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We report here eight new hominoid specimens, attributable to *Ramapithecus* and *Sivapithecus*^{1,2}, from the middle Miocene Chinji Formation of Pakistan. Hominoids previously described from the Chinji Formation cannot be precisely located although they most probably come from the upper two-thirds of the formation^{3–9}. The new material, from the middle portion of the formation, is ~12–13 Myr old based on magnetostratigraphy (refs 10, 11 and N. Johnson, personal communication). Two earlier specimens are supposedly from the underlying Kamli Formation^{6,12}, although the best candidate for an earliest *Ramapithecus* or *Sivapithecus* is an undescribed molar, AMNH 19565B, collected by Brown¹³ from a locality believed to be basal Chinji¹⁴, for which magnetostratigraphy indicates an age of 14.5 Myr (ref. 11 and N. Johnson, personal communication). Small hominoids are definitely known in the older Manchar Formation in Sind¹⁵. Together with the material discussed here, the new specimens record the earliest phases of Asian hominoid evolution following dispersal from Africa–Arabia¹⁶, and the apparent division of large hominoids into 'African' (lineages leading to *Pan*, *Gorilla* and *Homo*) and 'Asian' (leading to *Pongo*) clades at a minimum of 13 Myr^{17–19}, probably more.

Table 1 lists the newly discovered specimens, Table 2 the associated Chinji Formation mammal fauna. The partial maxilla, GSP 16075, comprises premaxillae, canine jugae, inferior portion of the nasal aperture, and both post-canine alveolar processes (Fig. 1a; Tables 1, 3). It is a *Sivapithecus indicus*, the type of which is supposedly from the Chinji Formation^{6,20}. Only the right canine and right first molar are represented by partial crowns, both quite worn. A right third molar represents the same individual. Judging from the size and shape of the roots, the incisor crowns were heteromorphic. Canines are outwardly rotated, the canine diastemas small, and canine fossas well developed. The molar enamel is thick. The maxillary sinus is present only over the molars. In these features GSP 16075 resembles younger *Sivapithecus* specimens from ~8-Myr late Miocene sediments near Khaur^{8,21} in the northern Potwar. Although the palate is damaged, it is clear that in GSP 16075 the procumbent naso-alveolar clivus terminates at a narrow incisive fossa recessed somewhat within the nasal cavity. Although damaged, this specimen resembles GSP 15000 and other younger *Sivapithecus* in these features, conforming to what has recently been described as an 'Asian' Miocene pattern¹⁷ also seen in *Pongo*.

Two small maxillary canines, GSP 17121 and 17122 (Fig. 1b), expand our knowledge of this tooth from the Chinji Formation, previously known from two larger^{5,6} specimens (GSI 192, 238) and a smaller one⁷ (YPM 13809). They probably represent males and females. Collectively, these canines differ from younger Siwalik canines: the mesial grooves are less well

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developed; crowns are lower; the crown-root junction is more sinuous, lingually extending superiorly; the roots taper to an apex that is more triangular; and crowns and roots are more 'flexed'. GSP 16075 canine roots, as determined by computerized tomography, resemble others from the Chinji Formation.

The two partial mandibles and an isolated P_4 also expand our knowledge of Siwalik hominoids. The smaller of the mandibles, 16077 (Fig. 1c), is a left corpus complete from midline to third molar. The tooth row would have shown marked divergence posteriorly, as do other small hominoids from the Potwar and elsewhere¹. The molars resemble *Ramapithecus punjabicus*, the type of which is said to be from the Chinji Formation^{6,20}. The symphysis has a long planum alveolare, well developed superior and inferior transverse tori, but no surface markings for digastric attachment. The corpus is shallow and robust, and the ascending ramus is situated >2 cm lateral to the posterior molars. The second and larger Chinji mandible, GSP 17125, is a right corpus, broken obliquely through the symphysis, lacking the tooth crowns but with the roots of the canine to the third molar. The symphyseal region and lateral buttressing resemble those of younger *Sivapithecus*¹.

Two kinds of hominoids, based mainly on size, can be recognized here and provisionally identified as *Ramapithecus punjabicus* and *Sivapithecus indicus*; the taxonomy and nomenclature of Siwalik hominoids are presently in flux. They resemble younger material assigned to these genera from the Potwar Plateau and from Haritalyangar and Ramnagar, India, in premaxillary, dental and mandibular features. However, there are differences, for example, in premaxillary length and maxillary canine morphology. In their basic 'Asian' patterns¹⁷, these Chinji hominoids differ from the older Kenyan and Ugandan material, although in some of the comparable features they do resemble a few other African middle Miocene hominoids from Fort Ternan, Majiwa, and Buluk^{22,23}.

The new postcranial specimens are described elsewhere²⁴. The cuneiform, GSP 17118, is morphologically and metrically similar to that of *Pan troglodytes* which implies functional

similarities in the foot. However, the incomplete capitate, GSP 17119, does not closely resemble any extant anthropoid (see Fig. 1d). Some of its features are unique and differ notably from those of extant Great Apes, including *Pongo*. Overall, the capitate implies that the hand was capable of extension and rotation in wide ranges of pronation and supination such as would be compatible with suspensory, climbing and palmigrade quadrupedal activities in an arboreal habitat.

This new material from Pakistan is important in recording significant facial, mandibular and postcranial features from known and dated geological contexts with abundant associated fauna. Large hominoids were part of middle Miocene South Asian faunas since at least 13 Myr ago as documented here, or more than 14 Myr if provenance of earlier specimens is correct. The main elements of that fauna may be 16 or 17 Myr old in South Asia, and it is possible that hominoids are of a similar age. The Siwalik hominoids differ from earlier Miocene African and many later Miocene European hominoids, as well as *Pan*,

Table 1 GSP-HPM hominoid specimens from the Chinji Formation

Specimen no. (GSP)	Description	Locality	Approximate age (Myr)
16075	Partial maxilla	494	13
17121	r C	496	13
17122	l C	496	13
16077	l mand. corpus	498	12-12.5
17125	r mand. corpus	499	12.5-13
17124	r P_4	496	13
17118	l lateral cuneiform	495	12
17119	r capitate	500	12.5-13

r, Right; l, left.

Table 2 Mammal species from the Chinji Formation near Chinji Village, Attock District, Pakistan

<i>Sivaladapis palaeindicus</i>	<i>Orycteropus</i> small sp.
<i>Sivapithecus indicus</i>	<i>Deinotherium pentapotamiae</i>
<i>Sivapithecus</i> (?) <i>simonsi</i>	' <i>Gomphotherium</i> ' <i>browni</i>
<i>Ramapithecus punjabicus</i>	Gomphotheriinae n. gen.
Sciuridae spp.	cf. <i>Stegolophodon</i> sp.
<i>Paraulacodus indicus</i>	<i>Choerolophodon</i> sp.
<i>Paraulacodus</i> n. sp.	' <i>Platybelodon</i> ' sp.
Thryonomyidae*	<i>Brachypotherium perimense</i>
<i>Sayimys sivalensis</i>	<i>Aprotodon fatehjangense</i>
<i>Sivacanthion complicatus</i>	<i>Chilotherium intermedium</i>
Gliridae*	<i>Gaindatherium browni</i>
<i>Kanisamys indicus</i>	<i>Chalicotherium salinum</i>
<i>Kanisamys potwarensis</i>	<i>Pecaricoerus orientalis</i>
<i>Democricetodon</i> , cf.	<i>Listriodon pentapotamiae</i>
<i>D. kohatiensis</i>	<i>Sanitherium cingulatum</i>
<i>Democricetodon</i> sp.	<i>Conohyus sindiense</i>
<i>Megacricetodon</i> spp.	<i>Hyotherium</i> (?) sp.
<i>Myocricetodon</i> spp.	<i>Lophochoerus</i> sp.
Cricetidae n. gen. and sp.	' <i>Anthracotherium</i> ' <i>punjabense</i>
<i>Antemus chinjiensis</i>	<i>Hemimeryx pusillus</i>
<i>Dissopsalis carnifex</i>	<i>Dorcabune anthracotherioides</i>
<i>Hyainailouros sulzeri</i>	<i>Dorcabune</i> small sp.
<i>Metapterodon</i> (?) n. sp.	<i>Dorcatherium minus</i>
' <i>Amphicyon</i> ' <i>palaeindicus</i>	<i>Dorcatherium</i> small sp.
<i>Agnotherium antigum</i>	<i>Dorcatherium</i> very small sp.
<i>Vishnuonyx chinjiensis</i>	<i>Giraffokeryx punjabiensis</i>
' <i>Martes</i> ' <i>lydekkeri</i>	<i>Palaeotragus</i> sp.
cf. <i>Ischyriactis</i> sp.	<i>Sivaceros gradiens</i>
<i>Sansanosmilus</i> sp.	<i>Protragocerus gluten</i>
<i>Percrocuta carnifex</i>	<i>Helicopotax tragelaphoides</i>
cf. ' <i>Miohyaena</i> ' <i>montadai</i>	<i>Sivoreas eremita</i>
<i>Protictitherium</i> (?) sp.	cf. <i>Elachistoceras</i>
' <i>Viverra</i> ' <i>chinjiensis</i>	<i>khauristanensis</i>
' <i>Sivaelurus</i> ' <i>chinjiensis</i>	<i>Kubanothragus sokolovi</i>
Felidae small sp.	<i>Caprotragoides potwaricus</i>
<i>Sivasmilus copei</i>	<i>Gazella</i> sp.

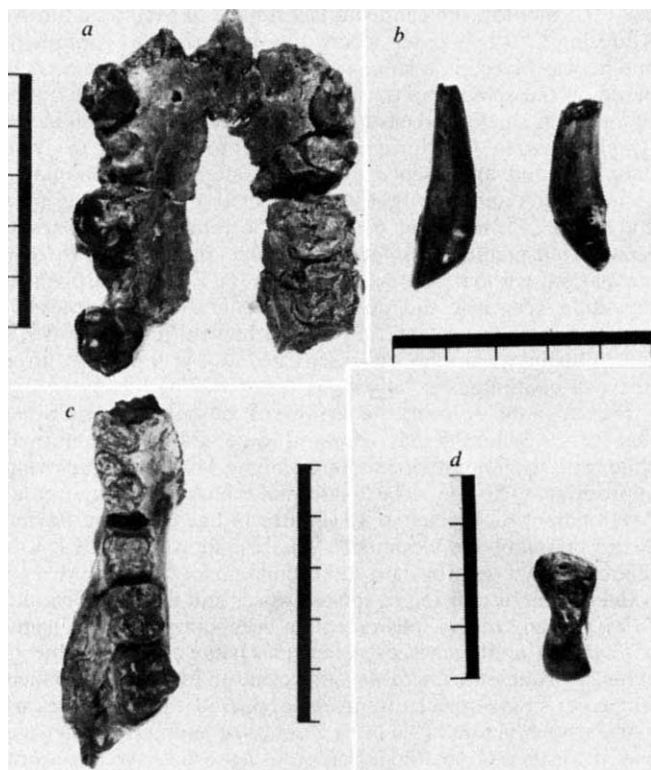


Fig. 1 a, GSP 16075, occlusal view; b, GSP 17121 and 17122, labial views; c, GSP 16077, occlusal view; d, GSP 17119, dorsal

Table 3 Facial and dental measurements (mm)

GSP 16075 (partial maxilla)	
Canine diastema r	3.7
l	3.0
Bicanine breadth	57.8
Inferior nasal breadth	22.5
Naso-alveolar clivus	25.8
GSP 17121 (C)	
md length	12.1
bl breadth	9.0
GSP 17122 (C)	
md length	12.4
bl breadth	9.0
GSP 16077 (partial corpus)	
M ₂ md length	12.0
M ₂ bl breadth	11.3
M ₃ md length	13.7
M ₃ bl breadth	12.4
Corpus height P ₃	26.5
Corpus breadth P ₃	13.7
Corpus height M ₃	19.8
Corpus breadth M ₃	22.5
GSP 17125 (partial corpus)	
Corpus height P ₃	38.5
Corpus breadth P ₃	16.2
Corpus height M ₃	30.6
Corpus breadth M ₃	24.2

r, Right; l, left; md, mesiodistal; bl, buccolingual.

Gorilla and *Australopithecus*. In some features the *Ramapithecus-Sivapithecus* material resembles the sole surviving Asian Great Ape, *Pongo pygmaeus*^{2,17-19,21,25}. We consider it likely that they represent part of a middle-late Miocene radiation of Asian apes, the only survivor of which is the orangutan. If correct, such an inference would calibrate the divergence of two hominoid lineages into proto-*Pongo* and proto-*Gorilla*, *Pan* and *Homo*, at least 13 Myr ago, probably more. At present, other hominoid lineage divergence times cannot be estimated nearly as well, although some cercopithecoid divergences can²⁶. As the Neogene catarrhine fossil record improves we can look forward to profitable collaboration between molecular and palaeontological primatologists concerning the timing of monkey, ape and human evolution.

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Language recovery following isolation for severe combined immunodeficiency disease

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Children with congenital severe combined immunodeficiency disease (SCID) lack an immune system and require continuous reverse isolation to protect them from exogenously transmitted infection. In our setting, protected environments consist of laminar flow rooms in which everything is sterilized (food, clothing, toys and so on). Staff and family wear complete surgical garb, preventing skin contact, observation of mouth movement and olfaction of typical human odours. Case studies of language development in children reared from infancy in protected environments have cited instances of mental retardation¹ or language delay with some improvements after intervention, although the degree of language exposure is not well documented²⁻⁵. We report here the case of a male patient, successfully treated for SCID, who lived in reverse isolation from 9 months to 4 yr 4 months of age and who has demonstrated nearly complete recovery of language usage. Our study illustrates the resilience of human intellectual function following a condition of early severe deprivation.

S.J., a male caucasian, was the 7 lb 9 oz product of an unremarkable full-term pregnancy. Although the first 5 months of life were uneventful, he then developed episodes of otitis media and respiratory tract infection. When he was 7 months old, S.J. developed pneumonitis. At 9 months he was placed in protective isolation for possible SCID in his home state.

At 9.5 months, the child was transferred to Memorial Sloan-Kettering Cancer Center where the diagnosis was confirmed, and he was placed in a laminar air-flow unit. The diagnosis was based on the absence of tonsillar tissues, palpable lymph nodes or a thymus shadow on chest X-ray; failure of peripheral blood lymphocytes to transform in response to mitogens or lymphocytes; and an absence of measurable levels of immunoglobulins IgA and IgG. IgM was present at a level of 5 mg ml⁻¹ and IgE at 24 U ml⁻¹ (ref. 6). During the initial year of isolation, he was transplanted with fetal liver tissue and received thymus irradiation, without success. A second fetal liver transplant in the same year also did not achieve engraftment. Successful bone-marrow transplantation was achieved just after S.J.'s fourth birthday, and he was discharged at 4 yr 4 months, after 3.5 yr of continuous isolation.

Nurses' notes, during the first week of isolation, indicated that S.J. (9.5 months old) appeared to be an otherwise normal child, with the amount of babbling, smiling, laughing and playing appropriate to his age⁷. The first formal evaluation of intellectual development took place at 18 months of age using the Bayley Scales of Infant Development⁸. The test showed that S.J. was capable of age-appropriate discrimination of visual form and could be taught to listen to spoken words and then to select the corresponding object. His receptive vocabulary included some body parts (mouth, ears, eyes, feet), his name and a few objects in his environment. There was, however, no evidence of spoken language; single-vowel utterances appeared to have no communicative function. This total absence of expressive language was in contrast to most children of his age who have one-word utterances for people, food, body parts, animals and clothing⁹. His most successful form of communication was a gestural system, using hands, and eye and head movements to indicate needs (for example, pointing to a toy). These gestures did not

appear to be symbolic in nature, but similar to movements usually associated with the paralinguistic part of normal spoken language.

A second evaluation using the Bayley Scales when S.J. was 21 months old showed that his receptive vocabulary was consistent with age expectation, but there was still no production of words: his word-like sounds (mixtures of vowels and consonants) still had no discernible linguistic relevance. English-speaking children attain a spoken vocabulary of 50 words between the ages of 15 and 24 months (ref. 9); S.J. did not speak at all. The gestural system remained unchanged, further suggesting its non-symbolic function.

A third evaluation was made at 52 months old, 2 weeks before discharge. At this point, he was producing some words ("yes", "no") and even stringing some words together (for example, "I want something else"). Conversely, the gestural system had nearly disappeared. S.J.'s receptive vocabulary was still confined to the few referents in his isolation unit. A similarly aged child reared in more normal circumstances has a vocabulary of more than 1,000 words and a language structure that includes the combination of two or more propositions in a single sentence, relative clauses and complements¹⁰, independent of culture¹¹.

The most recent language evaluation occurred at the age of 4 yr 9 months, 5 months after discharge from protective isolation. S.J. had recently been placed in a pre-school language programme in his home state. Test data and an analysis of audio recording taken during formal and informal testing revealed tremendous development in his language. He was able to identify (receptively and expressively) numbers, letters, shapes, familiar objects, primary colours, clothing, foods and body parts. He was able to respond to WH questions (who, what, where, when) and to make judgements using language concepts involving position, size and quantity. The Token Test for Children¹² showed receptive semantic and syntactic competence that matched expectations for his age. S.J. was also able to utter sentences with as many as seven or eight words ("I want to play with the sailboat"), although most of his productive language was expressed in phrases and sentences of two to four words. Nevertheless, his expressive language conveyed intentionality, with pronouns used to point out referents and to refer back to items in earlier discourse, and he was able to make assertions and negations (for example, Examiner: "When do you want to go home?" S.J.: "Today, I hope").

Despite this enormous progress, we identified weaknesses that required therapy. S.J. rarely initiated discourse, although he was capable of doing so. This deficiency may be related to the social deprivation associated with isolation with its enforced passivity for medical treatment, and therefore may concern the pragmatic aspect of language¹³. S.J. had been encouraged to speak during his stay in the laminar flow unit, but the lack of vocalization during his hospital stay and the low frequency of spontaneous expression afterwards suggest that his medical isolation had not provided a context to use language. In addition, his articulation was poor, and intense therapy is underway. A pure-tone hearing test showed a deficiency in the 6,000–8,000-Hz range, bilaterally. It is difficult to determine whether the hearing loss is the result of treatment during isolation or the earlier occurrence of otitis. There is no evidence that immune deficiency, *per se*, retards cognitive or linguistic development.

Other authors have suggested the adverse impact of severely impoverished environments on development and intellect^{14–17}. Because of the risk involved in restricting sensory and social input, not all transplant units isolate children for prolonged periods, despite immune deficiency¹⁸. There have been studies in which children were severely deprived of the opportunity to hear and practise speech: for example, the wild boy of Aveyron¹⁹ and Genie²⁰, a 13-year-old girl almost totally deprived of hearing speech for 11 yr by her parents. Nevertheless, we have demonstrated an instance in which a child has overcome such circumstances. Extreme isolation does not therefore lead inevitably to permanent intellectual dysfunction (but see refs 2, 3, 5).

Our patient was babbling at the time of admission and the

family history indicates that he probably had normal linguistic input until isolation, providing further evidence that early interactive patterns may be important for subsequent development^{21,22}. During isolation S.J. was exposed to some language, especially during meals, hygienic care and medical procedures; stimulation, however, was not consistent. He did play some games with his mother that did not require his vocalization. S.J.'s language recovery after discharge, despite the lag in linguistic development, is consistent with the hypothesis that a critical period for language extends to puberty¹¹. The absence of expression, however, does not preclude the possibility that there was some form of linguistic competence not assessed by the Bayley Scales. Further research will help to identify the critical variables that facilitate development in medically isolated children and provide insight into normal growth.

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Central serotonergic nerves project to the pial vessels of the brain

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Serotonin is strongly implicated in the aetiology of several cerebrovascular (circulatory) diseases, including stroke, migraine and vasospasm. Previous studies have suggested the existence of an indoleaminergic system of perivascular nerves in large cerebral arteries of the lamprey^{1,2}. In addition, some authors have observed that cerebral arteries (such as the vertebralbasilar system of the rabbit) and microvessels may take up serotonin and 5-hydroxytryptophan in various species^{3–6}. However, neither large cerebral arteries nor microvessels (primarily capillaries) directly control, or change, cerebral blood flow; as in other vascular beds, it is the arterioles and small arteries that are the major resistance elements. We report here on the presence of a central serotonergic innervation of pial arteries and arterioles in the rat, using immunocytochemical and neurochemical techniques. The fibres seem to have a central neuronal origin, emanating from both median and dorsal raphe nuclei. This perivascular serotonergic innervation may have a role both in the normal regulation of the cerebral circulation and in pathological conditions.

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Immunocytochemistry, using indirect immunofluorescence techniques and highly specific, well characterized antibodies towards serotonin, revealed the presence of perivascular, serotonin-containing fibres in the pial blood vessels of the rat (Fig. 1). The organization of the nerve plexuses resembles that of other vasomotor nerves observed previously in the pial vessels (noradrenaline-, acetylcholinesterase-, vasoactive intestinal polypeptide- and substance P-containing nerve fibres)⁷⁻⁹. In the major cerebral arteries (anterior, middle and posterior cerebral, cerebellar and basilar arteries) the serotonin-immunoreactive fibres formed plexuses with fibres running in all directions whereas smaller pial arteries had few or only one nerve fibre (Fig. 1).

Based on these histochemical findings, we have undertaken biochemical studies to confirm the existence of this putative serotonergic innervation. To obviate any contamination from blood elements, all animals were perfused before the removal of the tissue samples. The rats were anaesthetized (2.0% halothane), heparinized and perfused with heparinized saline via the left ventricle until the right atrial return was entirely clear. Substantial concentrations of serotonin and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) were found in the pial vessels when compared with cortical tissue (see Fig. 2 legend and Table 1).

To determine whether this serotonergic innervation was neuronal in origin, serotonin and 5-HIAA concentrations were measured 2 weeks after the intracerebroventricular administration of the neurotoxin 5,7-dihydroxytryptamine (5,7-DHT, 200 µg) to rats pretreated with desipramine (25 mg per kg intraperitoneally (i.p.)) and nomifensine (10 mg per kg, i.p.) to selectively destroy indoleaminergic nerve terminals. Administration of 5,7-DHT reduced the levels of serotonin and 5-HIAA in pial vessels by 68 and 72%, respectively (Fig. 2), while respective levels in the cerebral cortex were reduced by 79% and 77%. The lack of significant changes in the cortical

concentrations of noradrenaline (NA) (sham, 289 ± 12 ng g⁻¹; lesioned, 316 ± 13 ng g⁻¹) after 5,7-DHT demonstrates the selectivity of the chemical lesion. As 5,7-DHT is known to destroy serotonin-containing nerves by entering nerve terminals via the high-affinity uptake process¹⁰, these biochemical data fully support the presence of a serotonergic innervation of pial vessels.

Attempts have been made to define the origin of this perivascular serotonergic innervation. A dense plexus of noradrenergic terminals exists in brain vessels, the fibres mainly originating from the superior cervical ganglion¹¹. As serotonin-immunoreactive cells have been located in this ganglion¹², we have investigated whether these cells might contribute to the serotonergic innervation of pial vessels. Ganglionectomies were performed under 2% halothane anaesthesia; the rats were perfused 7 days later. Bilateral removal of the superior cervical ganglia failed to alter the pial vessel concentrations of serotonin and 5-HIAA (Table 1) whereas NA concentrations were reduced by 56% (sham, 2.22 ± 0.25 ng per mg protein; sympathectomized, 0.97 ± 0.05 ng mg⁻¹, $P < 0.001$).

These data indicate that the serotonergic innervation of pial vessels does not originate from the small, intensely fluorescent cells of the superior cervical ganglion and also rule out the possibility that serotonin is taken up and stored in noradrenergic terminals in cerebral vessels.

The ascending serotonergic innervation of the cerebrum originates from the raphe nuclei located in the brain stem¹³. To investigate the possibility that the pial vessel serotonergic innervation also arises from the raphe nuclei, the pial vessel concentrations of serotonin and 5-HIAA were measured after selective electrolytic lesioning of either the median or the dorsal raphe nuclei. Serotonin concentrations in pial vessels were reduced by 54 and 48% 2 weeks after destruction of the median and dorsal raphe, respectively (Table 1). Similar but less pronounced changes were observed for 5-HIAA. As the serotonergic innervation of the striatum and hippocampus originates from the dorsal and median raphe, respectively¹⁴, serotonin levels were also measured in these areas to assess the specificity of the lesions. Lesion of the dorsal raphe reduced serotonin levels by 51% in the striatum but failed to alter the amine levels in the hippocampus (Table 1). In contrast, lesion of the median raphe reduced serotonin levels by 68% in the hippocampus but failed to do so in the striatum. Electrolytic lesions of the raphe nuclei failed to alter serotonin concentrations in platelet-poor plasma (sham, 224 ± 40 ng ml⁻¹; dorsal raphe lesion, 290 ± 84 ng ml⁻¹; median raphe lesion, 207 ± 4 ng ml⁻¹), eliminating a non-

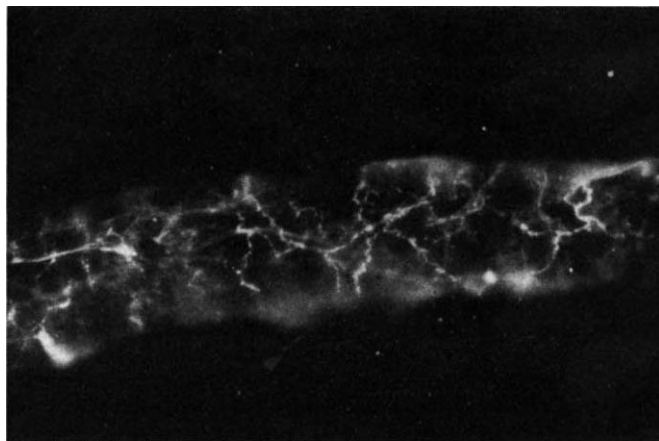


Fig. 1 Perivascular serotonin-containing nerve fibres in the walls of pial arteries. The animals were decapitated under diethyl ether anaesthesia, and the major arteries at the base of the brain and the pial membrane of the parieto-temporal cortex were dissected out. The specimens were spread flat on microscope slides as whole mounts and immersed in 4% formaldehyde buffer solution for 4 h, followed by rinsing in ethanol¹⁸. Subsequently, the indirect immunohistochemical procedure of Coons *et al.*¹⁹ was used. After incubation in 0.1 M phosphate-buffered saline (PBS) at room temperature for 30 min, the specimens were incubated at 4 °C for 18 h with serotonin antiserum. The serotonin antibodies used were those of Steinbusch and have been characterized in detail elsewhere^{12,18}. Less than 1% cross-reactivity was found with catecholamines. The specimens were then rinsed and treated with fluorescent isothiocyanate-conjugated sheep anti-rabbit IgG, diluted 1:16 with PBS containing 0.1% Triton X-100. After the final incubation the sections were rinsed in PBS and mounted under a coverslip in a mixture of glycerine-PBS (3:1, v/v). The sections were examined under a Zeiss microscope equipped with incident illumination for fluorescence. $\times 176$.

Table 1 Effect of sympathectomy or electrolytic lesion of dorsal or median raphe nuclei on concentrations of serotonin and 5-HIAA in rat pial vessels and brain areas

Surgical treatment	Serotonin (ng per mg protein)			5-HIAA (ng per mg protein)
	Pial vessels	Striatum	Hippocampus	Pial vessels
Sham	1.80 ± 0.34			2.97 ± 0.60
Sympathectomy	2.08 ± 0.26			2.94 ± 0.35
Sham	2.65 ± 0.31	6.31 ± 0.19	3.99 ± 0.12	2.24 ± 0.16
Dorsal raphe lesion	$1.38 \pm 0.15^*$	$3.08 \pm 0.12^*$	3.89 ± 0.32	$1.57 \pm 0.16^*$
Median raphe lesion	$1.22 \pm 0.18^\dagger$	5.62 ± 0.20	$1.30 \pm 0.33^\dagger$	$1.70 \pm 0.10^*$

Rats were killed 1 week after sympathectomy and 2 weeks after bilateral electrolytic lesion of either the dorsal or median raphe. Ganglionectomies were performed bilaterally under 2% halothane anaesthesia. Lesions were performed, under chloral hydrate (400 mg per kg, i.p.) anaesthesia, with a nichrome wire coaxial insulated electrode (0.5 mm diameter) set at an angle of 45° from the vertical axis. To lesion the dorsal raphe nuclei, the electrode was introduced successively at the following coordinates: AP 5, DV 5, L 0 and AP 6, DV 4.9, L 0 (atlas of König and Klippel)¹⁷ and a current of 3 mA was applied for 4 s. To destroy the median raphe nuclei, a current of 3 mA was applied for 3 s to an electrode located successively at the following coordinates: AP 3.4, DV 1.9, L 0 and AP 3.4, DV 2, L 0 (ref. 17). The location of the lesion was confirmed histologically. Animals were perfused with heparinized saline via the left ventricle, before decapitation. Serotonin and 5-HIAA concentrations in pial vessels removed bilaterally were assayed as described in Fig. 2 legend. Results are means $\pm$ s.e.m. for data obtained on 8–15 animals per group.

* $P < 0.01$, $^\dagger P < 0.001$ versus respective controls.

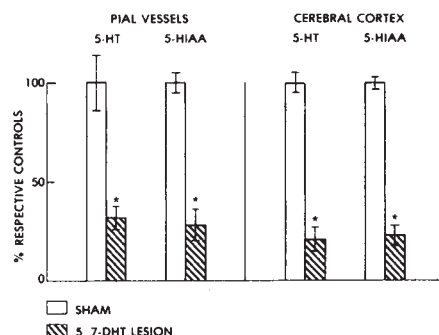


Fig. 2 Effect of 5,7-DHT treatment on serotonin (5-HT) and 5-HIAA concentrations in pial vessels and cerebral cortex of the rat. Male Sprague-Dawley rats (COBS CD strain from Charles River, France) weighing 150 g at the beginning of the experiment were used. 5,7-DHT (200 µg per 10 µl) was injected intracerebroventricularly (coordinates AP 5.4, DV 7.5, L 2; ref. 20) to rats pretreated with desipramine (25 mg per kg, i.p.) and nomifensine (10 mg per kg, i.p.) (to prevent destruction of catecholaminergic neurones) 2 weeks before the experiment. The brains were perfused *in situ* to remove blood elements before decapitation. The skull was opened and the adhering dura mater was removed. The pial vessels and arachnoid were simply and clearly removed bilaterally from the whole brain surface using fine forceps and iridectomy scissors (the pia mater and underlying cortex were not disturbed). A possible contamination of the pial vessels sample by the adjacent cerebral cortex is excluded due to the protective presence of the pia mater between the pial vessels and the cortical tissue. Moreover, the purity of the pial vessel sample has been confirmed by conventional histology. The mean weight of pial vessels removed from one rat for biochemical measurement was 1.3 ± 0.1 mg. Due to this low amount of tissue, results were preferably expressed as ng per mg protein. Cortical tissue and pial vessels were homogenized in 1 ml and 250 µl of 0.1 M perchloric acid, respectively. After centrifugation, serotonin and 5-HIAA were assayed on 100 µl of the supernatant (corresponding to ~0.5 mg of pial vessel tissue) by HPLC with electrochemical detection²¹. The limit of sensitivity of assay (signal-to-noise ratio of 2) was 10 pg for serotonin. In our assay conditions, the serotonin levels measured in pial vessel samples represented at least 10 times the limit of assay sensitivity. Results are means $\pm$ s.e.m. of data obtained for nine rats per group and are expressed as a percentage of related control values which were: (ng per mg protein) serotonin, pial vessels 2.67 ± 0.52 , cerebral cortex 3.11 ± 0.29 ; 5-HIAA, pial vessels 2.62 ± 0.27 , cerebral cortex 1.00 ± 0.11 . * $P < 0.01$ versus respective controls (Student's *t*-test).

specific depletion of pial vessel serotonin due to the lesion.

Taken together, these data indicate that most of the serotonergic innervation of the pial vessels originates from both median and dorsal raphé, both nuclei contributing equally to this innervation. However, the two ascending serotonergic pathways may project to two demarcated cortical regions; this possibility cannot be ruled out as the pial samples invested the entire cerebrum. The leptomeningeal distribution of mast cells is particularly rich; in the rat, the intracranial mast cells contain serotonin. However, the fact that selective raphé lesions decrease pial vessel serotonin concentrations would, almost certainly, rule out the possibility that this serotonin was located in perivascular mast cells¹⁵.

In conclusion, the present immunocytochemical and biochemical studies clearly indicate the existence of a serotonergic innervation of the pial circulation which has a largely central midbrain origin. This innervation provides a sound basis for a serotonergic coupling between cerebral function and perfusion. As cerebral vessels respond to exogenous serotonin in a dramatic and complex manner—both extreme vasoconstriction and marked arteriolar dilatation have been noted¹⁶—an indoleaminergic link between cortical activity, metabolism and bloodflow is entirely feasible. These serotonergic nerves may be involved in the physiological regulation of the cerebral circulation and, furthermore, may well be implicated in the aetiology of serotonin-related cerebrovascular disorders.

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Differential localization of type I and type II benzodiazepine binding sites in substantia nigra

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A number of studies have suggested the existence of multiple benzodiazepine binding sites in the brain^{1–20}. We have recently reported the physical separation of two apparent benzodiazepine binding site subtypes²¹, the pharmacological properties, and distribution in tissue sections of which correspond to the putative type I and type II sites^{20,22}. Benzodiazepine and γ -aminobutyric acid (GABA) receptors have been shown to interact²³, and lesions of the GABAergic striatonigral pathway, which lead to GABA supersensitivity^{24,25}, both increase the numbers of GABA binding sites^{24,26} and enhance GABA-stimulated benzodiazepine binding²⁷. We demonstrate here that degeneration of striatonigral fibres increases the density of putative type I benzodiazepine binding sites in the substantia nigra and decreases the density of the putative type II sites. This suggests that type I sites that increase after denervation are postsynaptic, whereas the type II sites reduced by the lesion may be localized to axons or terminals of the striatonigral pathways.

To visualize type II sites selectively, we evaluated ³H-flunitrazepam (³H-FNZ) binding in the presence and absence of 0.2 µM CL218872, a drug with a 50–100-fold greater selectivity for type I than type II sites²⁰ ($K_D = 20$ nM for type I sites²⁸, $K_i \sim 1,000$ nM for type II sites²⁹). At 0.2 µM concentration, CL218872 displaces about 83% of type I sites and only 10% of type II sites³⁰. To examine type I sites selectively, we treated brain slices with 2% sodium cholate. Type II sites are completely solubilized by this concentration of Triton X-100 or sodium cholate while type I sites are unaffected in biochemical studies²¹. Type I and type II binding site densities estimated from ³H-FNZ binding to brain slices in the presence or absence of 2% sodium cholate in a wide range of brain areas are identical to values obtained by displacement with CL218872 (ref. 22). We also labelled tissue sections with the tritiated ethyl ester of

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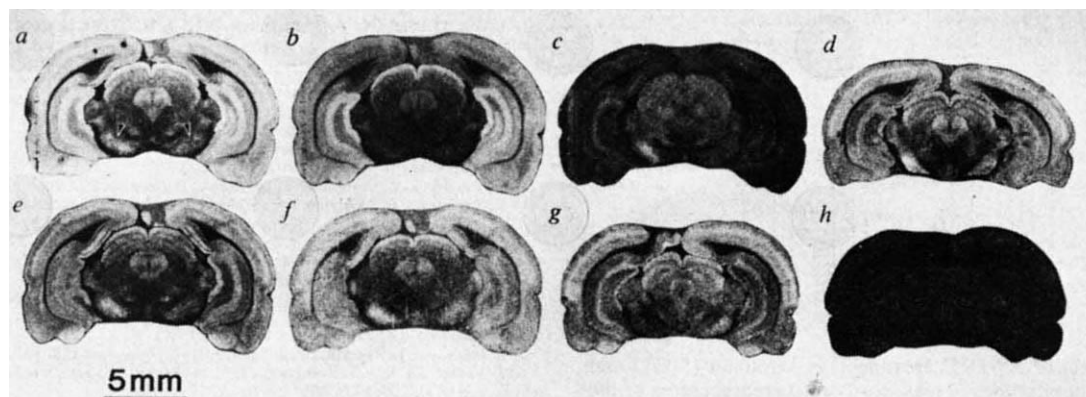


Fig. 1 Dark-field photomicrographs of coronal sections of rat brain showing substantia nigra (indicated by arrowheads in *a*) of lesioned (left) and control (right) sides. Sections *a* to *f* were caudate-lesioned brains after 13 days and *g* and *h* were substantia nigra-lesioned (2 μ g ibotenic acid in 1 μ l) brains after 11 days. Autoradiograms of ^3H -ligand binding are: *a*, ^3H -FNZ; *b*, ^3H -FNZ+CL218872; *c*, ^3H -FNZ in section pretreated with 2% sodium cholate; *d*, ^3H -ECC; *e*, ^3H -Ro 15-1788; *f*, ^3H -muscimol; *g*, ^3H -ECC; *h*, ^3H -ECC in the presence of 1 μM ECC. Nonspecific binding of ^3H -FNZ, ^3H -Ro 15-1788 and ^3H -muscimol constituted less than 1% of the total binding. Nonspecific binding of ^3H -ECC was ~30–40% of total binding.

β -carboline carboxylic acid (^3H -ECC) which has about 10-fold higher affinity for the putative type I than type II sites^{7,31,32} ($K_D = 0.5$ –1 nM for type I sites).

We used ibotenic acid to lesion striatonigral GABAergic fibres, as this destroys neurones intrinsic to the injected structure while sparing terminals and fibres of passage. Although ibotenic acid is about 10 times less potent than kainic acid, it causes a more uniform sphere of neuronal degeneration and is more reproducible in this effect³³. Sprague–Dawley rats (150–200 g) were given unilateral stereotaxic injections of ibotenic acid (1 μ g in 1 μ l saline) into the caudate-putamen. We compared the binding of ^3H -FNZ (a benzodiazepine agonist) and ^3H -Ro 15-1788 (a benzodiazepine antagonist³⁴) in the substantia nigra of the control and lesioned sides. Unlike flunitrazepam, Ro 15-1788 shows no interactions with peripheral-type benzodiazepine receptors³⁴. We also evaluated the binding of ^3H -muscimol as a ligand for GABA receptors.

With either ^3H -FNZ or ^3H -Ro 15-1788, destruction of striatonigral afferents with ibotenic acid elicits a 30–60% augmentation of total specific benzodiazepine binding in the substantia nigra (Fig. 1, Table 1). We also obtained a 70% increase in GABA receptor binding (Fig. 1, Table 1), as others have reported previously^{24,26}. The specificity of the changes in benzodiazepine binding sites observed following striatonigral lesion is emphasized by the absence of changes in ^3H -muscimol or ^3H -benzodiazepine binding in other brain areas such as cerebral cortex, dentate gyrus and cerebellum, or in substantia nigra of sham-lesioned animals. Examination of sagittal and coronal sections indicates that the augmentation of ^3H -Ro 15-1788 and ^3H -FNZ binding in the absence or presence of sodium cholate as well as ^3H -muscimol binding is greatest in the posterolateral aspect of the substantia nigra pars reticulata (Fig. 1).

Type I and type II sites in the substantia nigra respond quite differently to striatonigral denervation (Fig. 1, Table 1).

Table 1 Benzodiazepine and GABA binding in substantia nigra after lesion of caudate or substantia nigra

Ligand	Specific binding (fmol per mg protein)				% Control
	Control	Control	Lesioned	Lesioned	
	Sagittal	Coronal	Sagittal	Coronal	
Caudate lesion					
^3H -FNZ					
Total	218 $\pm$ 16	244 $\pm$ 18	362 $\pm$ 17†	353 $\pm$ 26†	155
+CL218872	108 $\pm$ 10	123 $\pm$ 9	86 $\pm$ 10	98 $\pm$ 4	
Cholate-treated	84 $\pm$ 5	117 $\pm$ 6	276 $\pm$ 24†	265 $\pm$ 15†	269
^3H -ECC	93 $\pm$ 4	94 $\pm$ 5	195 $\pm$ 10†	183 $\pm$ 14†	200
^3H -Ro 15-1788	313 $\pm$ 19	289 $\pm$ 19	396 $\pm$ 11*	378 $\pm$ 24*	129
^3H -Muscimol	296 $\pm$ 34	236 $\pm$ 25	523 $\pm$ 27†	369 $\pm$ 40†	168
Substantia nigra lesion					
^3H -FNZ					
Total		234 $\pm$ 21		164 $\pm$ 6†	68
+CL218872		110 $\pm$ 6		102 $\pm$ 5	
^3H -ECC		85 $\pm$ 8		41 $\pm$ 3†	49
^3H -Ro 15-1788		201 $\pm$ 7		168 $\pm$ 6*	84

Thirteen days after intrastriatal injection of 1 $\mu\text{g}/\mu\text{l}^{-1}$ ibotenic acid using stereotaxic coordinates given in ref. 24, rats were perfused intracardially with 0.32 M sucrose in 0.05 M phosphate buffer and brains were frozen in liquid N_2 . 8- μm cryostat sections in either the coronal or sagittal plane were subsequently incubated for 40 min at 0 °C with 1 nM ^3H -FNZ, 0.5 nM ^3H -Ro 15-1788 or 15 nM ^3H -muscimol, in 50 mM sodium potassium phosphate, pH 7.4, +1 mM EDTA, then washed and dried under cold, dry air. Blanks were generated by the inclusion of 1 μM clonazepam, 1 μM Ro 15-1788 or 10 μM GABA in the incubation medium. Autoradiograms were generated by the apposition with tritium-sensitive film³⁹. To differentiate subtypes of benzodiazepine receptor binding, serial sections were co-incubated with ^3H -FNZ and 0.2 μM CL218872 (ref. 28), 1 nM ^3H -ECC (nonspecific binding determined with 1 μM ECC) or by detergent extraction in 2% sodium cholate at 0 °C for 60 min followed by three 5-min washes before incubation in ^3H -FNZ. Quantification of optical density was performed using a scanning densitometer; optical density values were converted to molar densities by use of a standard curve derived from calibrated tritium standards containing known amounts of radioactivity, co-exposed with the autoradiograms³⁹. The optical density of the standards increased linearly with increasing radioactivity up to 80,000 d.p.m. per mg protein. All optical densities observed in this experiment fell within this linear portion of the calibration curve; hence only this portion of the curve was used to calculate molar density values. Values shown are the mean $\pm$ s.e.m. of five or six determinations in each of four to six different animals.

* $P < 0.05$, † $P < 0.02$, lesioned versus control nigra, Student's *t*-test.

Table 2 Type I and type II benzodiazepine binding sites in substantia nigra after lesions of caudate or substantia nigra

		Specific binding (fmol per protein)		sodium cholate treatment	
		CL218872 displacement			
Caudate lesion					
	Control side:	Type I	Sagittal 121 ± 21	Sagittal 84 ± 5	Coronal 117 ± 6
		Type II	97 ± 10	134 ± 11	127 ± 10
	Lesioned side:	Type I	329 ± 16†	276 ± 24†	265 ± 15†
		Type II	33 ± 4*	86 ± 8*	88 ± 9*
Substantia nigra lesion					
	Control side:	Type I	138 ± 15		
		Type II	96 ± 10		
	Lesioned side:	Type I	63 ± 7*		
		Type II	101 ± 12		

Denervated substantia nigra groups represent animals receiving ibotenate lesions in the caudate unilaterally while lesioned substantia nigra groups comprise rats with ibotenate injections unilaterally into the substantia nigra. Type I and type II sites were differentiated by comparison of ^3H -FNZ-labelled sections preincubated in 50 mM sodium potassium phosphate, 1 mM EDTA at 4°C for 60 min with serial sections either co-incubated with ^3H -FNZ and 0.2 μM CL218872 or preincubated in 2% sodium cholate. Molar densities were derived from optical densities as described in Table 1 legend. In the presence of 0.2 μM CL218872 and 1 nM ^3H -FNZ, 83% of the type I sites and 10% of the type II sites are blocked³⁰. Thus, total binding can be apportioned into type I and type II components by using the simultaneous equations:

Total binding = type I + type II

Binding displaced by 0.2 μM CL218872 = 0.83 type I + 0.10 type II

Values shown are the mean $\pm$ s.e.m. of five or six determinations in each of four to six different animals. The different control levels of binding in sagittal sections obtained with CL218872 or sodium cholate treatment may reflect different levels of the nigra used for these assays.

* $P < 0.05$, † $P < 0.02$, lesioned versus control nigra, Student's *t*-test.

Autoradiograms of ^3H -ECC binding and of ^3H -FNZ binding in the substantia nigra pretreated with sodium cholate show a pronounced increase in grain density on the denervated side compared with the control side. Quantification of benzodiazepine type I and type II sites in the posterolateral nigra indicates a 100–170% increase in type I site binding following the lesion (Table 2). The validity of the calculation of type I and type II sites is indicated by the similarity in values obtained for the two receptor subtypes in control and denervated nigra whether the numbers of putative receptor subtypes are derived from displacement of ^3H -FNZ binding by CL218872, from sodium cholate treatment or from ^3H -ECC binding.

In marked contrast to the pronounced augmentation of type I sites following striatonigral lesions, the lesions reduce type II sites (Table 2). This effect is not simply an artefact of calculating type II sites by subtracting type I binding from total binding following cholate treatment, because type II sites have also been determined directly by measuring residual ^3H -FNZ binding after protection of type I sites by CL218872. The pronounced opposite responses of type I and type II sites to striatonigral denervation further strengthens the biochemical studies²¹ that types I and II sites are separate molecular entities and not merely conformers of a single protein.

In biochemical studies of physically separated type I and type II sites, both subtypes are stimulated by GABA²¹. Chloride ions also enhance benzodiazepine binding in membranes^{35,36} and in solubilized and separated benzodiazepine binding site subtypes²¹. The chloride stimulation is restricted to type II sites, with no detectable enhancement of type I binding³⁷. Also, we have reported that ^3H -CL218872 binding in rat cerebellum, a selective measure of the type I sites, is unaffected by chloride²⁸. We evaluated the influence of 10 μM GABA and 100 mM NaCl on total ^3H -FNZ binding in control and lesioned nigra (data not shown). GABA enhancement of ^3H -FNZ binding is observed in both intact and lesioned nigra. On the other hand, while a 40% augmentation of ^3H -FNZ binding by NaCl occurs in the intact nigra, no augmentation is detected in the lesioned nigra. This result corroborates our other evidence that the striatonigral lesion reduces the binding levels associated with chloride-sensitive type II sites.

In one preliminary study, striatonigral fibre degeneration following unilateral striatal kainic acid lesions elicited a decreased affinity of ^3H -diazepam binding, with no change in B_{max} in homogenates of brain stem areas that include the substantia nigra³⁸. In that study the decreased endogenous GABA following the lesion may have produced the decreased affinity

of ^3H -diazepam binding, as endogenous GABA enhances the affinity of benzodiazepine binding. In another preliminary report²⁵, striatonigral degeneration induced by unilateral electrolytic lesion of the crus cerebri produced a modest reduction in ^3H -diazepam binding in homogenates of nigra-containing brain stem regions. As the dissected area in those studies includes a substantial amount of brain tissue other than substantia nigra, those results cannot be compared with the localized changes observed here within a discrete region of the pars reticulata of the substantia nigra.

If types I and II sites are indeed post- and presynaptic respectively to the striatonigral pathway, destruction of intrinsic nigral neurones should reduce type I but not type II sites. We performed unilateral lesions of the substantia nigra with ibotenic acid (2 $\mu\text{g } \mu\text{L}^{-1}$) to destroy cell bodies but spare nerve terminals or fibres. After 11 days a slight reduction in benzodiazepine binding (^3H -FNZ or ^3H -Ro 15-1778) is observed in lesioned compared with control substantia nigra (Table 1). In contrast, binding of the putative type I-selective drug ^3H -ECC is reduced by 50% (Table 1) and type II sites measured by displacement of ^3H -FNZ with CL218872 are not affected in the lesioned nigra (Table 2).

Biochemical properties of type I sites have implications for the subcellular localization of these apparently postsynaptic type I sites. The resistance of type I benzodiazepine binding sites to detergent solubilization suggests that they are a part of the cytoskeletal matrix²¹. In subcellular fractionation studies we have observed a marked enrichment of type I sites in purified postsynaptic densities, obtained by extensive fractionation of detergent-treated brain membranes (R. R. Trifiletti, M.M.S.L. and S.H.S., in preparation).

The reduction of type II sites following striatonigral lesions suggests that these sites are located at least in part on axon terminals of the striatonigral pathway. Whether they are contained on descending GABA or other neurones is unclear. We cannot rule out the possibility that the reduced binding of type II sites following the striatal lesion involves trans-synaptic effects. However, our finding that type II binding sites are significantly reduced within even 7 days of striatonigral lesion strongly supports their localization on striatonigral axons or terminals.

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Tonotopic organization of the auditory neuropile in the bushcricket *Tettigonia viridissima*

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Invertebrate central neural structures, especially sensory or motor neuropiles, have proved rather intractable to conventional physiological analysis as they are considered to have a diffuse or unordered structure. This is also true for the auditory neuropiles in the ventral nerve cord of orthopterans, where receptor fibres synapse to interneurons. In the bushcricket, *Tettigonia viridissima*, this neuropile constitutes the terminations of about 40 receptor fibres, which derive from chordotonal receptor cells in the tibia of the foreleg. Zhantiev¹ and Rheinlaender² have shown that each receptor cell is tuned to a 'best frequency'. Oldfield³ has further shown that the sensilla are organized tonotopically within the organ, the proximal and distal receptors being tuned to low and high frequencies respectively. Besides the finding that auditory receptor axons form a diffuse neuropile on each side of the prothoracic ganglion^{4,5}, there are no data on the projection of single receptor fibres within the neuropile. The results presented here show that the auditory neuropile of *T. viridissima* is tonotopically organized, extending to within the central nervous system the tonotopic organization of the peripheral sense organ.

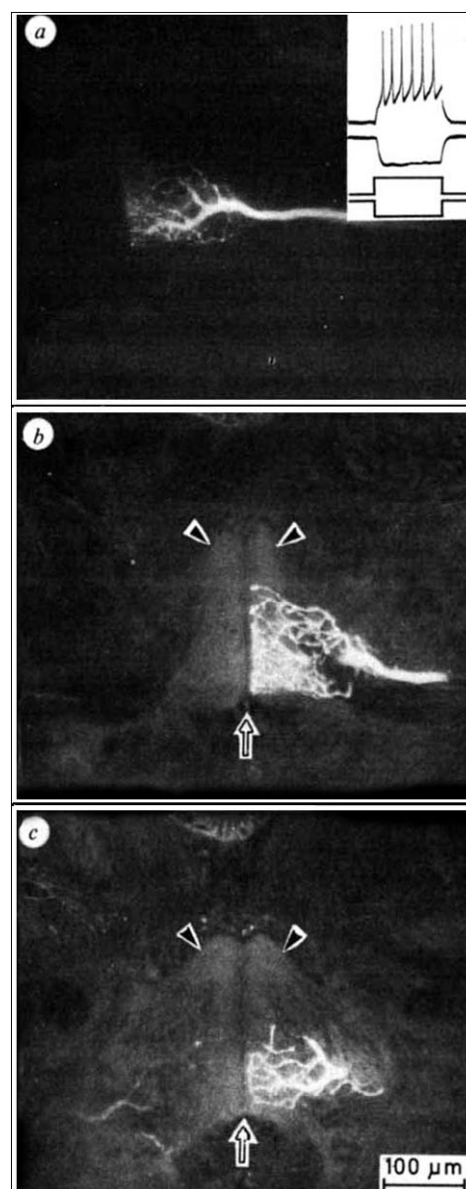


Fig. 1 Intracellular staining with Lucifer yellow of the 20-kHz receptor axon in the prothoracic ganglion of *Tettigonia viridissima*. **a**, Dorsal view of a whole-mount preparation: inset shows the electrophysiological proof of intracellular penetration using current pulses of 0.5 nA to depolarize (upper trace) and hyperpolarize (next trace) the axon membrane. **b** and **c** show two successive sections of the same preparation as in **a**. Note that the border of the auditory neuropile is visible only in sectioned material (arrow-heads). The midline is indicated by an arrow.

Intracellular recordings were made from auditory receptor axons in the prothoracic ganglion, close to where they branch within the neuropile (Fig. 1). Glass microelectrodes filled with Lucifer yellow (CH)⁶ (Sigma, type IV) were used. De- or hyperpolarization of the axonal membrane due to positive or negative current pulses was monitored to ensure that the microelectrode was positioned intracellularly (see inset of Fig. 1). A tuning curve and intensity-response characteristic were obtained from the fibre which was then filled with Lucifer yellow by passing negative current through the electrode.

The individual fibres were first identified in whole-mounts viewed under the fluorescence microscope. Figure 1 shows a preparation with the fibre tuned to 20 kHz. However, other structures, such as the border of the neuropile, which could be taken as a reference point for the mapping of the axon branches, are not visible in whole-mount preparations. The ganglia were therefore embedded in low-viscosity epoxy resin and cut

Fig. 2 Reconstruction of the projection areas within the auditory neuropile in two preparations (*a* and *b*), in which one fibre on each side of the prothoracic ganglion was stained with Lucifer yellow. The midline and border of the neuropile are indicated by broken lines. The corresponding tuning curve for each fibre is plotted on the right-hand side. Sound intensity is given in dB re $2 \times 10^{-5} \text{ N m}^{-2}$. The projection areas of fibres tuned to different frequencies differ in size and location within the neuropile.

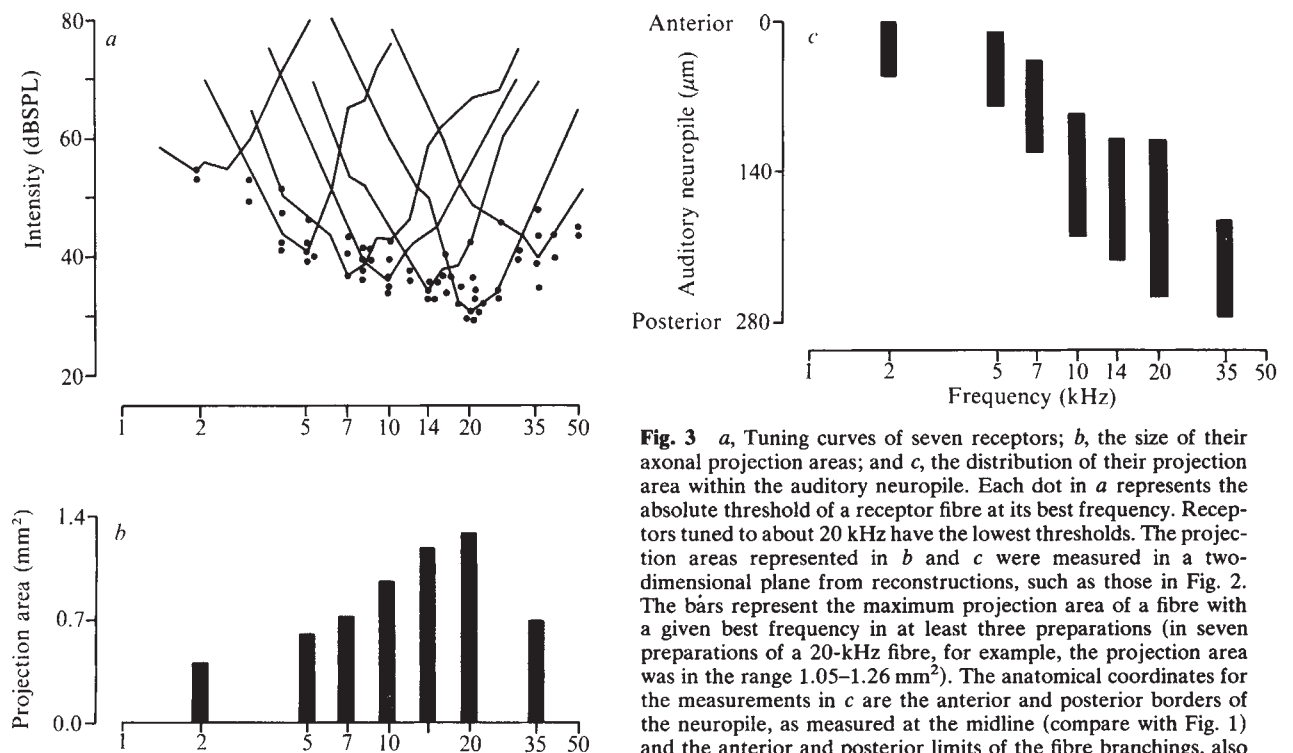
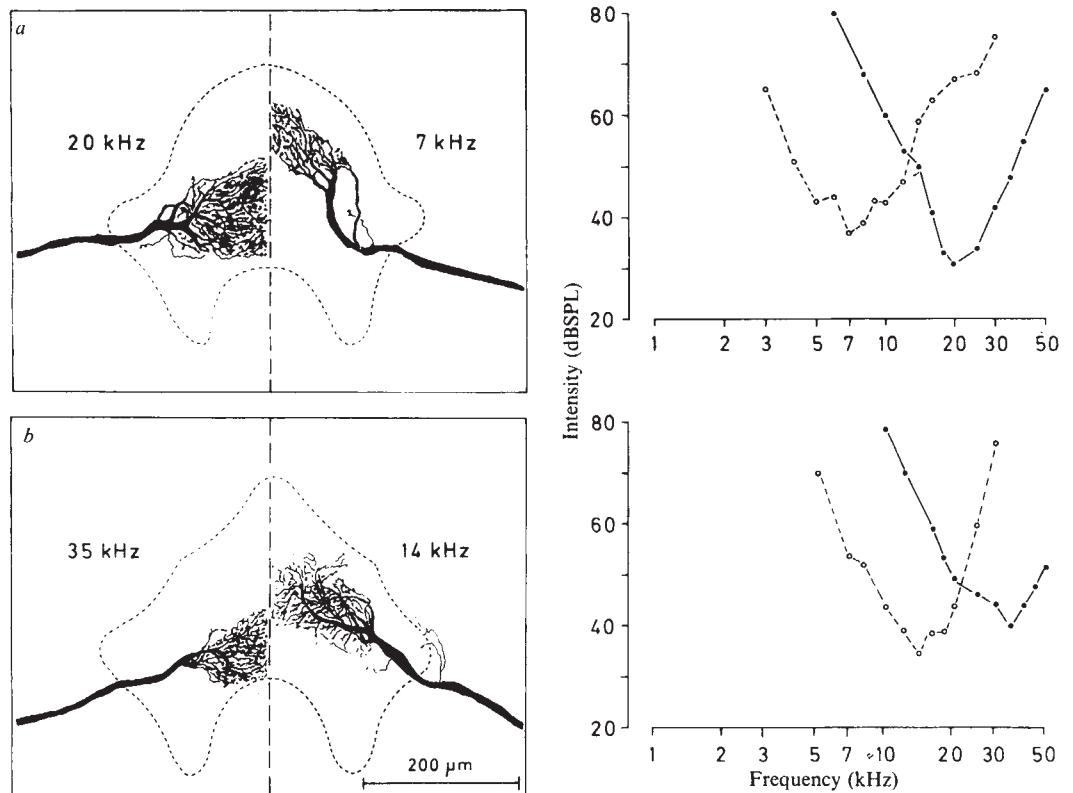


Fig. 3 *a*, Tuning curves of seven receptors; *b*, the size of their axonal projection areas within the auditory neuropile. Each dot in *a* represents the absolute threshold of a receptor fibre at its best frequency. Receptors tuned to about 20 kHz have the lowest thresholds. The projection areas represented in *b* and *c* were measured in a two-dimensional plane from reconstructions, such as those in Fig. 2. The bars represent the maximum projection area of a fibre with a given best frequency in at least three preparations (in seven preparations of a 20-kHz fibre, for example, the projection area was in the range 1.05–1.26 mm²). The anatomical coordinates for the measurements in *c* are the anterior and posterior borders of the neuropile, as measured at the midline (compare with Fig. 1) and the anterior and posterior limits of the fibre branchings, also measured at the midline. The results show that there is a spatial sorting of frequency (that is, tonotopy) within the neuropile.

horizontally, into 40 μm -thick sections. This greatly improves the resolution of the beaded fibre endings and allows the branching pattern of the axon to be reconstructed in relation to the border of the neuropile (Fig. 1*b, c*); 54 axons from 39 animals were prepared anatomically in this way. Figure 2 shows two cases in which the branching patterns of two receptor axons were traced, one on each side of the ganglion. The corresponding tuning curves for each pair of fibres are shown together on the

right of the figure. Figure 2*a* shows that the projection area of the 7-kHz receptor endings is smaller and lies anterior to that of the 20-kHz receptor. Similarly, Fig. 2*b* illustrates the 14-kHz receptor with a larger projection area than that of the opposite 35-kHz receptor which reaches the posterior border of the neuropile.

Figure 3 summarizes the tuning of the fibres, the size of their projection area and the distribution of their endings within the

neuropile for the 54 axons examined. The seven representative tuning curves, together with the best frequency threshold points of further fibres in Fig. 3a, illustrate how the overall sensitivity of the organ results from the composite response of about 40 individual receptors which are each tuned to a different frequency, the most sensitive being tuned to a frequency of ~20 kHz. A comparison of Fig. 3a and b shows that the tuning of the seven receptors in Fig. 3a is correlated with the size of their projection area, being centred about the 20-kHz receptor whose axon has the largest projection area. Furthermore, Fig. 3c shows that the projection areas are tonotopically arranged with respect to the anterior-posterior axis of the auditory neuropile so that fibres tuned to low frequencies project into the anterior part of the neuropile and those tuned to high frequencies project into the posterior part (see also Fig. 2). An overlap of the projection areas is most marked for the fibres tuned to within the 10–20-kHz range. Despite this, one can regard the auditory neuropile of *Tettigonia* as being tonotopically organized, thus preserving the structural and physiological organization of the receptor organ. This is analogous to the auditory system of vertebrates in which frequency is represented spatially in the organization of the cochlea and the brain⁷.

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The song of *T. viridissima* has a broad-band spectrum with several peaks including one at ~20 kHz, which is also the frequency at which directional sensitivity is optimal⁸. Moreover, the results presented here show that the receptor tuned to 20 kHz has the largest projection area within the auditory neuropile and thus potentially the largest field of synaptic influence. The significance of the 20-kHz frequency in the life of *T. viridissima* is therefore reflected in the organization of receptors in the auditory organ as well as in the central nervous system, which is similar to findings in vertebrates⁹.

Oldfield¹⁰ has confirmed that there is some degree of tonotopic organization in the Australian bushcricket *Caedicia simplex* using similar methods. Thus it seems that the use of intracellular recording and marking with Lucifer yellow, combined with histological methods for identifying nervous background structures, is appropriate for further study of sensory and motor neuropiles in invertebrates.

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Is extracellular calcium buffering involved in regulation of transmitter release at the neuromuscular junction?

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During synaptic activity at the neuromuscular junction, sodium, potassium and calcium ions flow through both the postsynaptic and presynaptic membrane^{1–4}. These ionic fluxes can cause changes in the local extracellular concentration in the synaptic gap: a decrease in the concentration of the inwardly flowing ions (sodium and calcium) and an increase in the outwardly flowing potassium ions⁵. To check whether depletion of calcium ions in the synaptic gap is involved in transmitter release, we have used calcium buffers to keep the extracellular calcium concentration almost constant. The expectation was that if depletion does occur, transmitter release will increase; if no depletion occurs, there will be no change in quantal release when the calcium concentration is the same in buffered and unbuffered bathing solutions. We report here that, surprisingly, perfusing the frog neuromuscular preparation with a calcium-buffered solution caused a decrease in transmitter release compared with that in an unbuffered solution with the same calcium concentration⁶. This presumably indicates that the calcium level in the synaptic cleft is higher than that in the bulk extracellular medium. If such a mechanism operates physiologically, it may provide an energetically economical way to determine the level of evoked transmitter release and thus synaptic efficiency.

Experiments were performed on the sartorius nerve-muscle preparation of the frog (*Rana ridibunda* and *Rana pipiens*). After dissection in standard frog Ringer solution, the prepara-

tion was perfused by low calcium (0.3–0.6 mM) Ringer containing 1–2 mM magnesium and 5 mM HEPES (pH 7). Calcium-buffered Ringer contained sodium citrate (5–10 mM introduced by substitution for sodium chloride). (This substitution ensured that all solutions contained 116 mM sodium while varying the ionic strength by no more than 6.5% and osmotic pressure by less than 3%.) In all experiments the nerve was stimulated at 0.5 Hz and synaptic activity was followed by conventional intracellular recording techniques. The signals were fed (after amplification) into a PDP 11/23 MINC microcomputer and analysed by FORTRAN and MACRO programs written for this purpose.

The calcium concentrations of all solutions were measured spectrophotometrically by murexide⁷, a dye suitable for measuring calcium in the concentration range used in these experiments, and electrochemically with calcium-specific electrodes⁸. The free calcium levels determined by murexide were generally higher and more reproducible than those measured by a commercial calcium electrode, so the values cited below rely on the murexide determinations. The somewhat lower calcium levels determined electrochemically may be due to interaction between citrate and the ion-exchanger membrane of the calcium-specific electrode. Such an interaction between other anions and calcium electrodes, leading to underestimates in calcium measurements, was reported recently⁹. In any case, even if we take the less reproducible data obtained by the calcium electrode, the results described below do not change qualitatively. Many molecules can be used as effective calcium buffers. We chose citrate because its maximal buffering capacity for calcium lies between 0.3 and 1.05 mM. Other investigators report that citrate binds calcium with a dissociation constant of 3.16×10^{-4} M (ref. 10) or 6.3×10^{-4} M (ref. 11); we obtained a value for the dissociation constant of 1.05×10^{-3} M in frog Ringer solution. These values (which differ by a factor of 3) all lie in the middle of the range where transmitter release strongly depends on extracellular calcium¹². Citrate also binds magnesium ions, with a dissociation constant of 4.17×10^{-4} M (ref. 10) or 6.3×10^{-4} M (ref. 11). Hence, the calcium-buffered Ringer must be prepared with appropriate total calcium and magnesium in order to obtain the desired free levels of these ions. In the presence of citrate, the extracellular free calcium level should remain at the value determined by

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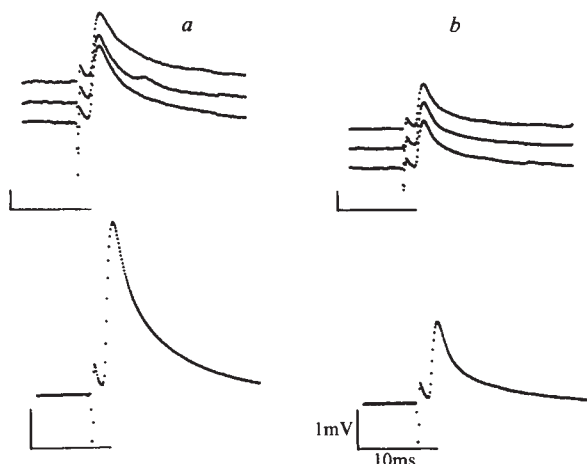


Fig. 1 Effect of calcium buffering on e.p.p. amplitude. *a*, Individual (top) and average (bottom) e.p.p.s in unbuffered Ringer containing 0.35 mM Ca + 1 mM Mg. *b*, Individual and average e.p.p.s in calcium-buffered Ringer containing 0.36 mM Ca + 1 mM Mg + 10 mM total citrate. Both solutions were proton-buffered at pH 7 by 5 mM HEPES. All recordings were collected from the same postsynaptic cell after a 40-min perfusion by each solution. Average responses represent 200 e.p.p.s evoked by stimulating the nerve at 0.5 Hz.

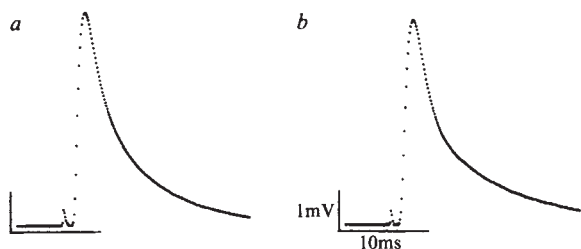


Fig. 2 Comparable e.p.p. amplitudes produced in calcium-buffered and unbuffered Ringer. Average responses (200 e.p.p.s) in unbuffered 0.35 mM Ca + 1 mM Mg Ringer (*a*) and buffered 0.61 mM Ca + 1 mM Mg Ringer (*b*).

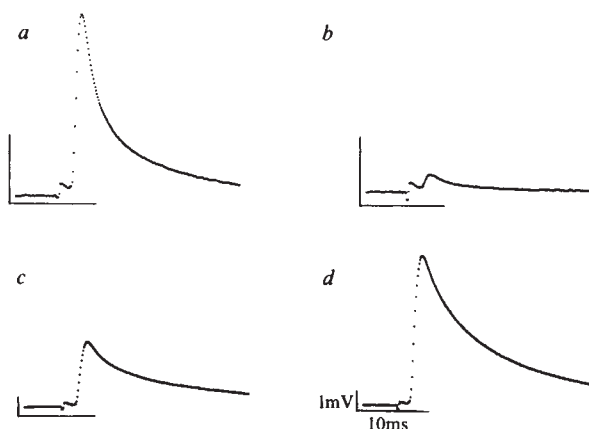


Fig. 3 Effect of increasing magnesium on e.p.p. amplitude in calcium-buffered and unbuffered Ringer. *a*, Average e.p.p. in an unbuffered solution containing 0.4 mM Ca + 1 mM Mg. *b*, Average e.p.p. after adding 2.57 mM Mg to the solution of *a*. *c*, Average e.p.p. in citrate calcium-buffered Ringer containing 0.6 mM free Ca + 1 mM free Mg. *d*, Average e.p.p. after introduction of an additional 2.57 mM Mg into the solution of *c*. Total calcium, magnesium and citrate concentrations were 2.37 mM, 5.93 mM and 10 mM, respectively, in *c*; 2.37 mM, 8.5 mM and 10 mM in *d*. Records *a* and *b* were taken from one cell, *c* and *d* from another cell.

this calcium buffer, overcoming possible local depletions or elevations in concentration.

Figure 1 illustrates average endplate potentials (e.p.p.s) recorded from the same postsynaptic cell in the presence of an unbuffered Ringer solution (Fig. 1*a*) and in a calcium-buffered Ringer solution of similar free calcium concentration (Fig. 1*b*). If calcium depletion occurs during such an experiment, it is expected that the e.p.p. amplitude in Fig. 1*b* will be larger than that in *a*. However, the opposite effect was observed: a striking reduction in e.p.p. amplitude and quantal content occurred in calcium-buffered solutions relative to unbuffered Ringer; the magnitude of the effect was between 50 and 90% (20 experiments with $[Ca] = 0.3\text{--}0.6$ mM). This is a presynaptic effect, as the amplitude of the quantum of transmitter release, measured from the miniature e.p.p. amplitude, is practically unaffected (see ref. 13).

To obtain similar e.p.p. amplitudes with and without citrate, it was necessary to increase the calcium activity in the buffered solutions, as shown in Fig. 2. Ten experiments with two different calcium activities indicate that the free calcium level in the buffered solutions must be elevated 1.5–2-fold to achieve amplitudes comparable with those in unbuffered Ringer. This may imply that when the nerve-muscle preparation is perfused by unbuffered Ringer, the calcium level within the synaptic cleft may be 1.5–2-fold higher than in the bulk solution. The structures responsible for this elevated free calcium are unknown, although fixed negative charges somewhere within the cleft¹⁴ or local extrusion mechanisms are likely candidates. Obviously, in the presence of calcium buffers, the cleft calcium concentration should be similar to that of the bulk and hence transmitter release will decrease.

The fact that citrate binds both calcium and magnesium ions enabled us to contrive an experiment to demonstrate further the difference in transmitter release in calcium-buffered and unbuffered Ringer. Figure 3*a, b* illustrates the effect of an increase in magnesium on e.p.p. amplitude in unbuffered Ringer. This well known reduction in amplitude^{15,16} is probably due to increased competitive binding of magnesium to calcium sites in the presynaptic membrane¹². In contrast, addition of magnesium to calcium-buffered Ringer results in the opposite effect, as shown in Fig. 3*c, d*. We interpret the latter effect by assuming that on addition of magnesium to a solution originally equilibrated with given amounts of calcium, magnesium and citrate, a redistribution of free and bound species of citrate occurs according to the Le Chatelier principle¹⁷: as the newly introduced magnesium binds to citrate, some of the calcium–citrate complex dissociates; the net result—an increase in free calcium—is reflected in the experiment as an enhanced e.p.p. amplitude (Fig. 3*d*) due to increased transmitter release.

The main calcium-buffering action of citrate solutions is probably extracellular. Passive penetration of the negatively charged citrate into the presynaptic cell is highly unlikely. Even if it did occur, such passive diffusion is expected to reduce intracellular free calcium by no more than 2%. Of course, we cannot rule out the possibility of active penetration, but we do not know of the existence of such a mechanism at the presynaptic membrane. Furthermore, citrate-loaded liposomes do not cause a reduction in e.p.p. amplitude. Such a procedure with other ions shows that the content of the liposome is transferred to the cell interior¹⁸. Therefore, if there is a pharmacological action of citrate other than on the extracellular calcium concentration, it does not seem to be on intracellular structures. Finally, preliminary experiments with another calcium-buffering agent, disodium phosphate, show that in this case, too, e.p.p. amplitudes are reduced, which indicates that the effect is not citrate specific.

The present experiments therefore suggest that the presynaptic nerve terminal 'sees' a higher calcium level than that present in the bulk solution. The factor(s) responsible for this local extracellular calcium elevation may have a key role in determining synaptic efficiency. If this non-homogeneity is caused by high density of negative charges or by highly localized extrusion processes, it may provide a mechanism to increase effectively

the electrochemical gradient for calcium across the presynaptic membrane and thus increase the number of quanta released by the nerve impulse.

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Involvement of guanine nucleotide-binding protein in the gating of Ca^{2+} by receptors

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The introduction of impermeant aqueous solutes into individual cells by microinjection has long been established^{1,2} but the difficulties of manipulating the cytosol composition of large populations of microscopic cells have only recently been overcome. Successful techniques include a dielectric breakdown procedure^{3–6}, treatment with micromolar concentrations of ATP^{4-} (ref. 7) and also with very small (that is non-agglutinating, non-fusogenic) amounts of Sendai virus⁸. So far, attention has been concentrated on the behaviour of the cells (generally their response to applied Ca^{2+} buffers) at the time when the membrane permeability lesions are open, and thus cytosol and external medium are in contact. I now report a novel technique for monitoring the state of molecular solute permeability in cell membranes and show that the lesions generated by ATP^{4-} in the membrane of mast cells can be closed within seconds of adding Mg^{2+} so that a cycle of permeabilization and resealing can be used to explore the effect of foreign compounds trapped in the cytosol of effectively intact cells. I show that non-hydrolysable GTP analogues, introduced into the cytosol of mast cells, cause them to undergo exocytotic secretion in response to addition of extracellular Ca^{2+} . This finding is discussed in the light of previous experience relating guanine nucleotide regulatory proteins as intermediaries between receptors and the transducers which they control.

The nuclear stain ethidium bromide is normally impermeant and fluorescent only when in contact with nucleic acid, and so can be used as a probe to determine the presence of membrane permeability lesions. Figure 1 shows the increase in fluorescence due to addition of ATP (3 μM) to mast cells suspended in a buffered solution containing the dye (25 μM) and salts of monovalent cations only (thus $\text{ATP}^{4-} = \text{ATP}_{\text{total}}$). The increase in fluorescence, which commences within seconds of applying the ATP, indicates that the dye is entering the cells and coming

into contact with the nuclear DNA. The penetration of the dye stops abruptly following addition of Mg^{2+} (1 mM) indicating that the membrane lesions close rapidly following complexation of the agonistic ATP^{4-} with the divalent cation. Further evidence that the lesions reseal on complexation of ATP^{4-} comes from the finding that the normally impermeant dye 6-carboxyfluorescein introduced into the cytosol by treatment with ATP^{4-} (ref. 7) becomes trapped following addition of Mg^{2+} ; this of course requires the isolation and extensive washing of the cells to remove the fluorescent dye from the exterior. Fluorescence measurements using 3,3'-diethylthiadicarbocyanine iodide⁹ indicate that the membrane potential, which is abolished on addition of ATP^{4-} , recovers during the 10 min following closure of the lesions with Mg^{2+} . The cells may subsequently be depolarized on addition of extracellular K^+ (75 mM).

The ethidium bromide technique for monitoring permeability lesions can be applied to other cell types and to other permeability-inducing agents. Thus both mast cells and rabbit neutrophils fluoresce following treatment with digitonin (2.5 nM) and with Sendai virus (25 haemagglutinating units ml^{-1}). Both these agents are believed to cause discrete membrane lesions in a wide variety of cell types without disruption of cell structure^{10,11}. The onset of fluorescence following addition of the virus is typically delayed by ~2 min.

Although the cells become unresponsive to subsequent stimulation by normal agonists if presented with ATP^{4-} at excessive concentrations (>10 μM) or over prolonged periods (>10 min)^{12,13}, they can tolerate a 5-min exposure to 3 μM ATP^{4-} if they are then incubated in the presence of excess Mg^{2+} for 10 min. Such cells recover a fairly normal pattern of response to stimulation with antigen, compound 48/80 or Ca^{2+} ionophores. The protocol for a typical loading experiment is therefore as follows: $t=0$, ATP (3 μM) added to mast cells suspended in a solution containing the solute to be loaded, and buffered at pH 7.7; divalent cations absent (EGTA 15 μM). $t=5$ min, Mg^{2+} (2 mM) added (the cells can be washed and the extracellular medium changed at this stage as required). $t=15$ min, cells transferred to tubes containing Ca^{2+} , agonists and so on as required. $t=25$ min, cells quenched by addition of ice-cold saline and secreted products measured as previously described⁸.

Figure 2a illustrates the dependence on extracellular Ca^{2+} of histamine secretion from cells which have been loaded according to the standard protocol with three concentrations of GppNHp. By comparison with the normal IgE-induced response, the requirement for Ca^{2+} in these cells is shifted to higher concentrations (around half of a log unit). Figure 2b shows a similar shift in Ca^{2+} dependence for antigen-stimulated histamine secretion from cells which have undergone the cycle of permeabilization and resealing in the absence of GppNHp. The shift is therefore due to effects of the permeabilization which would permit entry of other solutes including Na^+ , Cl^- and the buffering ions and also some leakage of nucleotides and other metabolic intermediates¹⁴.

Figure 3 illustrates the dependence of secretion on the loading concentration of three non-hydrolysable analogues of GTP [loading concentrations exceed the final intracellular concentrations to an unknown extent since equilibration of solute uptake by mast cells treated with 3 μM ATP is far from complete by 5 min (ref. 7): the relative uptake of the three analogues of GTP used in this experiment is unlikely to differ significantly]. The $-\gamma\text{S}$ (GpppS) derivative was the most active of the compounds tested, both in causing the cells to secrete in response to extracellular Ca^{2+} and in inhibiting the response at supra-optimal concentrations. Most of the experiments have been carried out using the $\beta\gamma$ -imido derivative. There was no effect of GppNHp on mast cells in experiments in which ATP was omitted from the first stage of the incubation, or in which Mg^{2+} was present from the start. The importance of the permeabilization step is shown further by an experiment in which cells were examined by fluorescence microscopy after treatment with ATP (1.25 μM) together with GppNHp and the dyes 9-aminoacridine

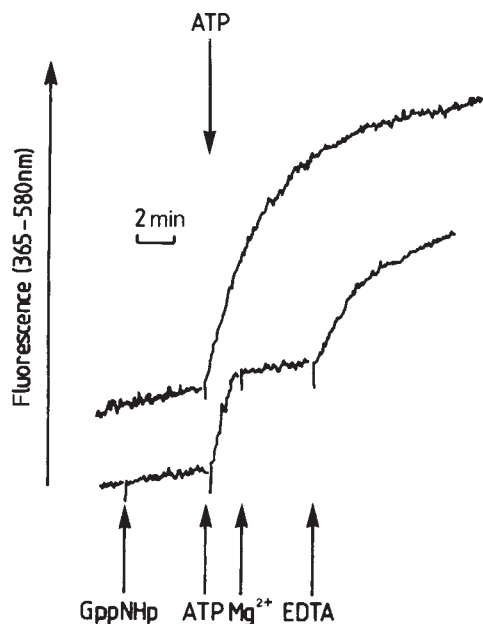


Fig. 1 Reversibility of the ATP^{4-} -induced lesions in the mast cell membrane: use of a novel general method for demonstrating molecular solute permeation in cells. Mast cells ($\sim 0.25 \times 10^6 \text{ ml}^{-1}$), isolated and purified as previously described¹², were suspended in a divalent cation-free salt solution (pH 7.7, comprising 137 mM NaCl, 2.7 mM KCl, 20 mM HEPES, 5.6 mM glucose, and 1 mg ml^{-1} bovine serum albumin). The cells were added to an equal volume of solution containing ethidium bromide (50 μM) in a cuvette maintained at 37 °C and provided with a magnetic stirrer (Rank, Cambridge). Fluorescence was measured at 365–580 nm using a Perkin Elmer model LS5 luminescence spectrometer. On addition of ATP (5 μM), the signal increased abruptly due to formation of the ethidium–DNA fluorochrome, demonstrating permeation of the cells by the normally impermeant dye. In the lower trace, 1 mM GppNHp (a synthetic analogue of GTP) has no effect on the uptake of the dye by itself, nor does it affect the ability of ATP (5 μM) to promote dye uptake. Addition of 1 mM Mg^{2+} halts dye uptake because of closure of the ATP^{4-} -induced lesions. The trace also demonstrates the reopening of the lesions on addition of excess EDTA, which, by complexing Mg^{2+} , ensures the presence once again of the agonistic ATP^{4-} .

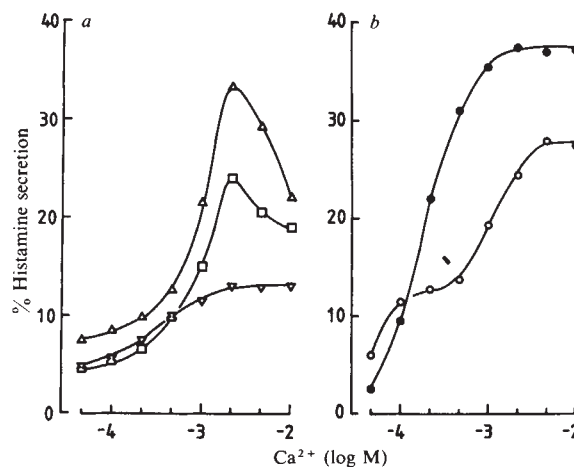


Fig. 2 Dependence on Ca^{2+} of histamine secretion from mast cells: *a*, loaded with GppNHp at three concentrations; *b*, resulting from stimulation with antigen. *a*, Purified mast cells in divalent cation-free buffered salt solution were treated at 37 °C with ATP (3 μM) and GppNHp at $10^{-4.25}$ (∇), $10^{-3.75}$ ($\square$) and $10^{-3.25}$ (Δ) M; after 5 min Mg^{2+} was added to 2 mM. After a further 10 min, the cells (50 μl) were transferred to tubes containing Ca^{2+} to give concentrations as indicated. Ten min later the reactions were quenched with ice-cold phosphate-buffered saline and the cells sedimented by centrifugation in the cold. Secreted histamine was measured as previously described¹². Secretion from control cells, permeabilized and resealed but not loaded with GppNHp, was <5% at all concentrations of Ca^{2+} . *b*, Mast cells, as a component of mixed peritoneal cells, were passively sensitized by incubation for 60 min with 1 $\mu\text{g ml}^{-1}$ mouse IgE hybridoma anti-DNP³⁴ (gift of Dr H. Metzger). The mast cells were isolated from unbound antibody and from other cell types by centrifugation through a cushion of bovine serum albumin (35%) as previously described¹². The cells were then incubated in divalent cation-free buffered salt solution, with or without ATP (3 μM) and Mg^{2+} (2 mM) added after 5 min. In this experiment, phosphatidyl serine (25 μM) was added to the cells 1 min after addition of Mg^{2+} to potentiate the secretion induced by stimulation of the IgE system³⁵. After a further 9 min the cells were added to tubes containing DNP–bovine IgG ($10^{-2} \mu\text{g ml}^{-1}$) and Ca^{2+} at the concentrations indicated. Secreted histamine was measured as described for *a*. All data points indicate means of duplicate determinations. Open symbols indicate secretion due to antigen stimulation after permeabilization with ATP and resealing as described. Closed symbols indicate secretion due to antigen stimulation of control cells.

(normally permeant and which selectively illuminates the low-pH environment of the secretory granules¹⁵) and ethidium bromide. Following the normal protocol for stimulation, only those cells having stained nuclei, and which had therefore been successfully permeabilized, had degranulated in response to subsequent addition Ca^{2+} . The method used to load the cells relies on the quite striking specificity of the ATP^{4-} receptor in this system¹²: it is quite distinct from the purinergic (P_1 and P_2) nucleotide receptors which give partial or total responses to ADP and AMP¹⁶. Neither ADP, AMP, nor any of the guanine nucleotides tested as intracellular effectors increased the rate of uptake of ethidium bromide even when present at 1 mM, nor did they affect dye uptake caused by ATP^{4-} at micromolar concentrations (Fig. 1, lower trace).

Secretion induced by internalized GppNHp was selective: in a representative experiment the release of lactate dehydrogenase did not rise above the basal level (11% in the absence of GppNHp) while release of histamine and β -N-acetylglucose aminidase increased in a similar manner dependent on Ca^{2+} up to maxima of 55% and 60% respectively as the concentration of GppNHp was increased in the range $10^{-4.5}$ – $10^{-3.25}$ M. As with other agonists, secretion does not occur from metabolically inhibited cells (treatment with 5 μM antimycin A plus 5 mM 2-deoxyglucose at the time of adding Mg^{2+}), nor from cells which have been previously heated at 45 °C for 15 min (neither treatment had any discernible effect on the rate of ethidium

bromide uptake due to ATP^{4-}). Secretion induced by internalized GTP analogues differs from that arising from stimulation of the IgE system in two ways: secretion is slow, extending over ~ 20 min, and it is unaffected by the presence of exogenous phosphatidylserine.

Other compounds which were tested in place of GppNHp include AppNHp, 5'-AMP, ADP, GTP, GDP, 5'-GMP, cyclic AMP and cyclic GMP. Of these, only GTP caused comparable responses though this was erratic and did not always occur. A small degree of Ca^{2+} -dependent secretion requiring permeabilization by ATP^{4-} did occur following application of $>300 \mu\text{M}$ 5'-GMP. When cyclic AMP was included at seven different concentrations (1–100 μM), together with three stimulating concentrations of GppNHp introduced at the time of permeabilization and isobutylmethylxanthine (1 mM) at the time of Mg^{2+} addition to prevent possible degradation of internalized cyclic AMP, it had no effect on the GppNHp-induced secretion. Cyclic GMP was tested in a parallel experiment and was also without effect on secretion. By contrast, GDP (in the range 10^{-4} – 10^{-3} M) acts competitively to inhibit secretion when loaded together with GppNHp.

It is worth considering the effects of incorporated GTP analogues in the light of knowledge of other activation systems in general and the mast cell in particular. In the mast cell, only one 'early event' in the stimulus–response sequence has been unequivocally identified: that elevation of cytosol Ca^{2+} into the

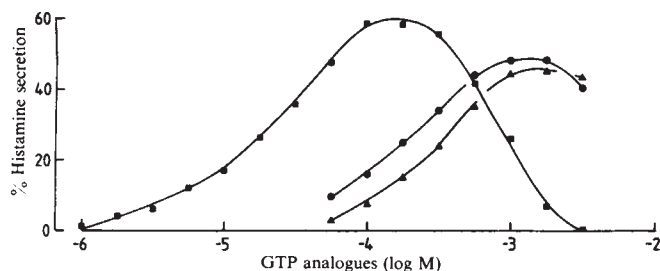


Fig. 3 Dependence of histamine secretion from permeabilized-resealed mast cells on the loading concentrations of three GTP analogues. Purified mast cells in divalent cation-free buffered salt solution were added to tubes containing equal volumes of a solution containing ATP (6 μ M) and GTP analogues (at twice the concentrations indicated). Mg^{2+} was added to 2 mM after 5 min, and Ca^{2+} was added to 5 mM after a further 10 min. The cells were quenched and secreted histamine measured as described for Fig. 2. ■, GTP γ S; ●, GppNHP; ▲, GppCH₂p.

micromolar range is the sole necessary precursor of secretion¹⁷. Many other metabolic events have been proposed to precede the increase in cytosol Ca^{2+} and in view of the present results the report of an early 'spike' in the concentration of cyclic AMP is pertinent¹⁸. Indeed, on the basis of parallel pharmacological modulation of the cyclic AMP spike and the extent of secretion by adenosine analogues, it has been argued that this is an obligatory precursor step controlling Ca^{2+} entry¹⁹. Activation of adenylate cyclase in all systems tested is known to involve the binding of GTP to specific guanine nucleotide-binding proteins which mediate the interaction between receptors and the catalytic unit²⁰. It is possible that the role of GTP analogues in the present experiments is to allow the activation of mast cell adenylate cyclase and hence permit entry of Ca^{2+} . But in this case, it is surprising that cyclic AMP had no effect on the subsequent Ca^{2+} -induced secretion in GppNHP-loaded cells.

An alternative possibility is that the Ca^{2+} channels of mast cells are regulated by their own class of guanine nucleotide-binding proteins. This is supported by an earlier report of mast cell activation by fluoride ion²¹, an effector of some guanine nucleotide-binding proteins²², which was not subject to modulation by agents known to affect cyclic nucleotide concentrations²³.

Guanine nucleotide-binding proteins also have roles independent of activation of adenylate cyclase. Thus in the regulation of protein chain elongation, factors Ts and Tu are analogous to the receptor and the guanine nucleotide-binding protein²⁴; in phototransduction in retinal cells, cyclic GMP phosphodiesterase is dependent on GTP and a guanine nucleotide-binding protein²⁵. Furthermore, it has recently been proposed that insulin affects a plasma membrane kinase and cyclic AMP phosphodiesterase through a specific guanine nucleotide regulatory protein^{26,27}.

Through the guanine nucleotide-binding proteins (which could act both to stimulate and to inhibit activation), receptors which control Ca^{2+} entry would share several important features with the above systems. Most important, the receptor itself (in the mast cell, the IgE receptor which becomes activated following cross-linking by suitable ligands) would not come into immediate contact with the transducer (the Ca^{2+} channel). Such a system would allow for control, in particular receptor desensitization which could occur by GDP binding to the guanine nucleotide-binding protein. The mechanism proposed for Ca^{2+} mobilization in mast cells may be a general one: in view of the observation that insulin, for example, exerts some of its effects through a guanine nucleotide regulatory mechanism^{26,27}, the present mechanism could explain how insulin triggers Ca^{2+} fluxes²⁸.

It has become customary to regard the receptors that cause activation of adenylate cyclase as quite distinct in their organization from those which act to mobilize Ca^{2+} (ref. 29). The present findings however support an earlier idea (expressed in very general terms, and which predated knowledge of the

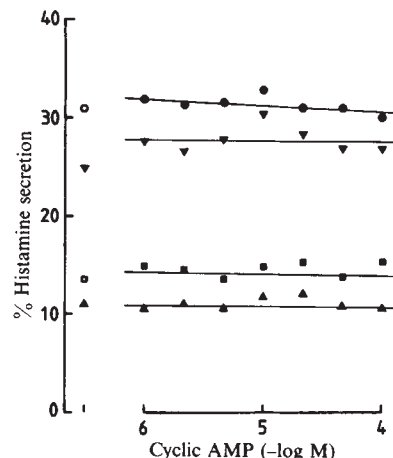


Fig. 4 Insensitivity to cyclic AMP of histamine secretion from GppNHP-loaded mast cells. Purified mast cells in divalent cation-free buffered salt solution were added to tubes containing equal volumes of ATP (6 μ M) plus cyclic AMP and GppNHP at twice final concentrations. Further treatment of the cells was as for Figs 2, 3. The lines linking the points were indicated by least-squares linear regression analysis of the data (zero cyclic AMP points omitted) according to the semilogarithmic presentation ($n = 14$ for each data set). GppNHP concentrations: ▲, 0; ■, $10^{-4.5}$ M; ▼, $10^{-4.0}$ M; ●, $10^{-3.5}$ M.

involvement of guanine nucleotides) for a unitary mechanism of action³⁰. Indeed, the possibility that guanine nucleotides may be involved in the action of a Ca^{2+} -mobilizing receptor has already been suggested³¹ by the observation that the affinity of ligand binding at muscarinic receptors and the *N*-formyl-methionyl peptide receptors of human neutrophils³² can be modulated by GTP³³.

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Release of Ca^{2+} from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5-trisphosphate

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Activation of receptors for a wide variety of hormones and neurotransmitters leads to an increase in the intracellular level of calcium. Much of this calcium is released from intracellular stores but the link between surface receptors and this internal calcium reservoir is unknown. Hydrolysis of the phosphoinositides, which is another characteristic feature of these receptors¹⁻³, has been implicated in calcium mobilization¹. The primary lipid substrates for the receptor mechanism seem to be two polyphosphoinositides, phosphatidylinositol 4-phosphate (PtdIns4P) and phosphatidylinositol 4,5-bisphosphate (PtdIns4,5P₂), which are rapidly hydrolysed following receptor activation in various cells and tissues⁴⁻¹⁰. The action of phospholipase C on these polyphosphoinositides results in the rapid formation of the water-soluble products inositol 1,4-bisphosphate (Ins1,4P₂) and inositol 1,4,5-trisphosphate (Ins1,4,5P₃)^{9,11,12}. In the insect salivary gland, where changes in Ins1,4P₂ and Ins1,4,5P₃ have been studied at early time periods, increases in these inositol phosphates are sufficiently rapid to suggest that they might mobilize internal calcium¹². We report here that micromolar concentrations of Ins1,4,5P₃ release Ca^{2+} from a nonmitochondrial intracellular Ca^{2+} store in pancreatic acinar cells. Our results strongly suggest that this is the same Ca^{2+} store that is released by acetylcholine.

The effect of inositol phosphates on intracellular Ca^{2+} stores was studied using isolated rat pancreatic acinar cells with permeable plasma membranes as described previously¹³. The plasma membrane was permeabilized by washing cells with a nominally Ca^{2+} -free solution. Cells were then incubated in a high-potassium solution with respiratory substrates and ATP. Ca^{2+} uptake or release from intracellular Ca^{2+} stores was determined by measuring the changes in free Ca^{2+} concentration of the incubation medium with a Ca^{2+} -specific macroelectrode¹⁴. In these conditions, intracellular organelles of the leaky cells take up Ca^{2+} from the medium until a steady state is reached at an external free calcium concentration of $\sim 0.4 \mu\text{mol l}^{-1}$ (Fig. 1). Subsequent addition of Ins1,4,5P₃ (isolated from red blood cell ghosts as described in Table 1 legend) resulted in an increase of medium $[\text{Ca}^{2+}]$ followed by re-uptake to the original steady-state concentration (Fig. 1). In the absence of cells, the addition of Ins1,4,5P₃ produced only a small increase of medium $[\text{Ca}^{2+}]$. This Ca^{2+} contamination of Ins1,4,5P₃ accounted for less than 5% of the effect shown in Fig. 1 and was corrected for in the numerical data. If cells were preincubated with the Ca^{2+} ionophore A23187 ($10 \mu\text{mol l}^{-1}$) to deplete intracellular Ca^{2+} stores, addition of Ins1,4,5P₃ resulted in the same small increase of medium $[\text{Ca}^{2+}]$ as observed in the absence of cells. These results show that the increase in medium $[\text{Ca}^{2+}]$ following addition of Ins1,4,5P₃ in the presence of cells is indeed due to Ca^{2+} release from membrane-bound cellular Ca^{2+} stores.

The possibility that calcium might be derived from the release of secretion granules was excluded by the observation that no amylase was detected following stimulation with either carbachol¹³ or Ins1,4,5P₃ (this study). To exclude the possibility that calcium release might be due to contaminants introduced

during the preparation of Ins1,4,5P₃, some of the red blood cell ghosts were taken through the complete preparative procedure (see Table 1 legend) except that they were incubated in calcium-free conditions to prevent the formation of inositol phosphates. Control samples prepared in this way failed to release calcium from pancreatic acinar cells.

The Ins1,4,5P₃-induced release of calcium showed a steep dose-response relationship with a clear effect at concentrations as low as $0.2 \mu\text{M}$ and a near-maximum release at $5 \mu\text{M}$ ($4.3 \pm \text{s.e.}$ $0.5 \text{ nmol per mg protein}$, $n = 16$; Fig. 2). A double reciprocal plot yielded an apparent K_m of $1.1 \mu\text{M}$ (Fig. 2, inset). Weiss *et al.*⁷ have shown that the breakdown of PtdIns4,5P₂ in stimulated parotid cells is of the order of $0.3 \text{ nmol per mg protein per min}$. This would give an intracellular concentration of Ins1,4,5P₃ of $\sim 30 \mu\text{M}$ which is in excess of the doses required to release calcium in these permeabilized cells.

The specificity of the calcium-releasing response was studied by testing the effect of Ins1,4P₂, inositol 1-phosphate (Ins1P), inositol 1,2-cyclic phosphate (cyclic IMP) and *myo*-inositol; none of these substances was able to release Ca^{2+} from intracellular stores (Table 1). When tested at the higher concentration of $5 \mu\text{mol l}^{-1}$, Ins1,4P₂ induced release of $\sim 10\%$ as much calcium as did the control (Ins1,4,5P₃), which can be fully accounted for by Ins1,4,5P₃ contamination of Ins1,4P₂.

If the Ins1,4,5P₃ sample was hydrolysed at 100°C for 30 min in 5 M HCl (conditions which randomize the phosphates by bond migration^{11,15}), its potency to release Ca^{2+} at a concentration of $1 \mu\text{M}$ decreased to $< 50\%$ of the control (100°C , 30 min, no acid). Thus, although quantitative conclusions cannot be drawn from these observations, they clearly demonstrate that the calcium-releasing activity depends on the distribution of phosphates on the inositol ring.

In a previous study on leaky acinar cells, it was shown that Ca^{2+} was taken up both by mitochondria and by at least one nonmitochondrial ATP-dependent Ca^{2+} store¹³. Mitochondrial Ca^{2+} uptake could be abolished by a combination of the mitochondrial inhibitors antimycin A ($10 \mu\text{mol l}^{-1}$) and oligomycin ($5 \mu\text{mol l}^{-1}$), whereas nonmitochondrial Ca^{2+} uptake was completely inhibited by high concentrations of the ATPase inhibitor vanadate (2 mmol l^{-1})¹³. Two separate calcium stores have also been identified in rat¹⁶ and guinea pig¹⁷

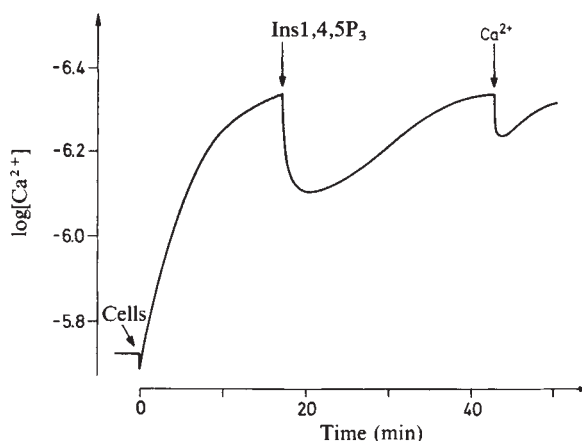


Fig. 1 Effect of Ins1,4,5P₃ on intracellular Ca^{2+} stores of leaky acinar cells. Isolated acinar cells from rat pancreas were prepared as described elsewhere¹³. Their plasma membrane was permeabilized by washing with a nominally Ca^{2+} -free solution (Trypan blue uptake about 70%). Cells (6.9 mg protein) were added to 3 ml of the following solution (mmol l^{-1}): 110 KCl, 6 MgCl₂, 5 K-succinate, 5 K-pyruvate, 25 HEPES, 5 K₂ATP, 10 creatine phosphate, 10 U ml⁻¹ creatine kinase, pH 7.4, at 25°C . The medium $[\text{Ca}^{2+}]$ was recorded continually with a Ca^{2+} -specific macroelectrode (neutral carrier ETH 1001). Electrodes were made and calibrated as described before^{13,14}. Ins1,4,5P₃ (see Table 1 legend) to a final concentration of $2.5 \mu\text{mol l}^{-1}$ and CaCl_2 (10 nmol) were added where indicated. This trace is representative of a typical experiment that was repeated at least 20 times.

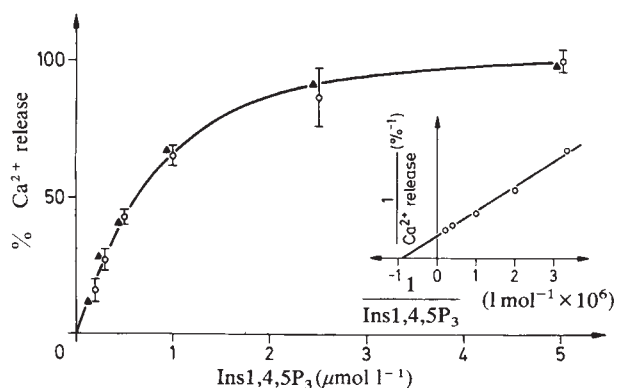


Fig. 2 Dose-response relationship of Ins1,4,5P₃-induced Ca²⁺ release. Ins1,4,5P₃-induced Ca²⁺ release was determined by comparison to known Ca²⁺ additions as described in Fig. 1 legend in either the standard incubation medium (○; mean ±s.e. of three different cell preparations) or the standard incubation medium supplemented with antimycin (10 μmol l⁻¹) and oligomycin (5 μmol l⁻¹) (▲; one experiment). Inset: double-reciprocal plot of the data without inhibitors.

Table 1 Calcium release by inositol phosphates and the effects of carbachol and metabolic inhibitors

Additions	Ca ²⁺ release (% of Ins1,4,5P ₃ control)	
Ins1,4,5P ₃ (5 μM)	100	(n = 12)
Ins1,4P ₂ (1 μM)	2 ± 1	(n = 4)
Ins1P (5 μM)	3	(n = 2)
Cyclic IMP (5 μM)	3	(n = 2)
myo-Inositol (100 μM)	0	(n = 2)
Ins1,4,5P ₃ (5 μM) + antimycin A (10 μM) + oligomycin (5 μM)	103 ± 11	(n = 4)
Ins1,4,5P ₃ (5 μM) + vanadate (2 mM)	42 ± 10	(n = 5)
Ins1,4,5P ₃ (5 μM) + carbachol (40 μM)	57 ± 4	(n = 4)

Calcium release was determined as described in Fig. 1 legend. The amount of calcium released is expressed as a percentage of that released by Ins1,4,5P₃ using the same cell preparation. The inhibitors antimycin A, oligomycin and vanadate were present in the incubation medium before addition of cells (about 20 min before addition of Ins1,4,5P₃). In the carbachol experiment, cells were stimulated with carbachol about 10 min before addition of Ins1,4,5P₃ as shown in Fig. 3. Results are expressed as mean ±s.e. Ins1,4,5P₃ and Ins1,4P₂ were prepared by incubating human red blood cell ghosts with CaCl₂ followed by a Dowex-formate column separation, and desalted by elution from a Dowex-Cl column with 1M LiCl, followed by removal of the LiCl with ethanol¹⁸. Ins1P and cyclic IMP were prepared by ionophoretic separation¹⁹ of the products resulting from enzymatic hydrolysis of yeast PtdIns¹⁹ by a rat brain²⁰ or celery²¹ soluble supernatant.

pancreas after permeabilization with saponin or digitonin. To identify which intracellular calcium store was sensitive to Ins1,4,5P₃, we compared the effect of these inhibitors on calcium release in our system. Incubation of cells in the presence of mitochondrial inhibitors did not impair the ability of Ins1,4,5P₃ to release Ca²⁺ (Table 1). The dose-response curve to Ins1,4,5P₃ was similar both in the presence and in the absence of antimycin and oligomycin (Fig. 2). This strongly suggests that Ins1,4,5P₃ does not release Ca²⁺ from mitochondria. In contrast, a clear, though not complete, inhibition was observed with the ATPase inhibitor vanadate, which inhibits Ca²⁺ uptake and thus reduces the amount of calcium in the nonmitochondrial pool (Table 1). Which of the nonmitochondrial pools is sensitive to inositol phosphates is not yet clear however.

Leaky cells retain the ability to react to carbamylcholine (carbachol) with Ca²⁺ release from intracellular stores¹³. It was possible, therefore, to test the interaction of the release mechanisms sensitive to carbachol and Ins1,4,5P₃. Addition of carbachol, like Ins1,4,5P₃, results in Ca²⁺ release from leaky cells

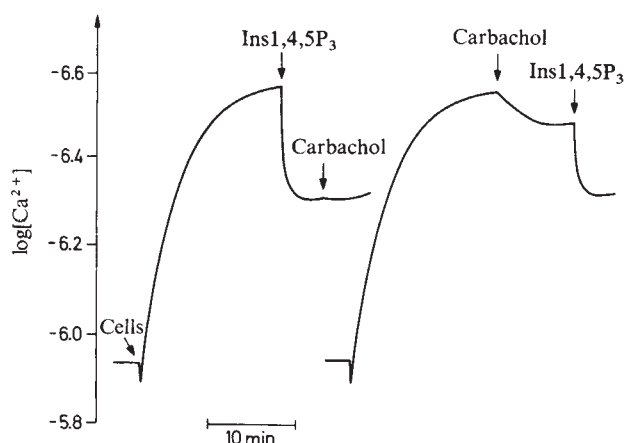


Fig. 3 Effect of sequential additions of Ins1,4,5P₃ and carbamylcholine. Isolated cells (2.5 mg protein ml⁻¹) were prepared and incubated as described in Fig. 1 legend. Where indicated carbachol (final concentration 40 μmol l⁻¹) or Ins1,4,5P₃ (final concentration 5 μmol l⁻¹) were added. Typical for four determinations.

followed by re-uptake to the prestimulation value¹³. If carbachol (40 μmol l⁻¹) was added after Ins1,4,5P₃ (5 μmol l⁻¹), the carbachol-induced Ca²⁺ release was completely abolished (Fig. 3). If, on the other hand, Ins1,4,5P₃ was added after carbachol, the Ins1,4,5P₃-induced release of calcium was decreased by about one-third (Fig. 3, Table 1). The sum of the Ca²⁺ released by carbachol and Ins1,4,5P₃ was constant. Thus, in the presence of saturating concentrations of exogenous Ins1,4,5P₃, carbachol can no longer release Ca²⁺, suggesting that both agents are acting on the same pool of releasable calcium. This in turn indicates that the carbachol-induced release of Ca²⁺ from the intracellular stores ('trigger Ca²⁺ pool') of these permeabilized pancreatic cells may be mediated by Ins1,4,5P₃.

A characteristic feature of calcium-mobilizing receptors is that they induce a rapid decline in the polyphosphoinositides⁵⁻¹⁰ with a corresponding increase in the levels of Ins1,4,5P₃ and Ins1,4P₂ (refs 9, 11, 12). Despite the probable relationship between this phosphoinositide effect and calcium signalling, the link between these two events has been missing. Our observations reported here may provide this missing link by suggesting that Ins1,4,5P₃ functions as second messenger to mobilize internal calcium. Whether phosphoinositides also regulate the permeability of the plasma membrane remains to be determined.

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Severe plasmalogen deficiency in tissues of infants without peroxisomes (Zellweger syndrome)

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The Zellweger syndrome is a lethal hereditary disease characterized by the absence of peroxisomes (microbodies) in liver and kidney, and variable abnormalities in mitochondria^{1–8}. We show here that tissues from five infants that had died of this syndrome contain less than 10% of the normal levels of phosphatidylethanolamine plasmalogen (pPE), a major phospholipid component of cellular membranes. Heart and muscle, but not other tissues, also contain a substantial fraction of phosphatidylcholine plasmalogen (pPC), and this fraction is also strongly reduced in the Zellweger patients. No other abnormalities in cellular phospholipids were detected. Key enzymes of the biosynthesis of plasmalogens have previously been shown to be exclusively located in the peroxisomes of rodent liver and the microperoxisomes of rodent brain^{9,10}. We infer that the corresponding enzymes are also located in peroxisomes in man and that the absence of peroxisomes in Zellweger patients leads to their inability to synthesize plasmalogens. Our results support the notion¹¹ that the biosynthetic role of peroxisomes in mammals has thus far been underestimated. We suggest that the defect in plasmalogen synthesis and possibly as yet unknown peroxisomal reactions are responsible for the diverse abnormalities observed in Zellweger patients.

Clinically the cerebro-hepato-renal syndrome of Zellweger is an autosomal recessive inborn error of metabolism, characterized by craniofacial malformations, a lack of muscle tone, disturbances in liver function, renal cysts and mental retardation. Patients usually die in the first year of life. In 1973 Goldfischer *et al.*¹ found two subcellular abnormalities in Zellweger patients: peroxisomes were absent in liver and kidney, and the mitochondria of liver and brain looked abnormal and seemed defective in electron transport. Studies on additional patients have essentially confirmed these findings^{3,5,7} and led to the identification of additional defects in the metabolism of pipecolic acid² and bile salts⁴.

Until recently, peroxisomes were thought to play only a modest role in mammalian metabolism, mainly involving production and degradation of H₂O₂ (refs 2, 12, 13). Hence, explanations for the serious defects in Zellweger patients have tended to focus on the mitochondrial defects^{1,3,7}. Recently, however, Hajra and co-workers^{9,10} found that some of the enzymes required for the introduction of ether bonds in alkylphospholipids are located in the peroxisomes of guinea-pig liver and the microperoxisomes of rat brain. The end-products of ether-phospholipid biosynthesis in mammals are mainly plasmalogens (alkenyl glycerol ether components). We have therefore determined the plasmalogen content of tissues of five Zellweger patients. The results are presented in Table 1

and, in summary form, in Fig. 1. In most tissues, the major plasmalogen found in non-Zellweger controls is phosphatidylethanolamine plasmalogen, but in heart muscle substantial amounts of phosphatidylcholine plasmalogen are also found. Both types of plasmalogens are nearly absent in Zellweger patients. The decrease in the plasmalogen fraction is balanced by increased amounts of phosphatidylethanolamine rather than non-plasmalogen alkyl ether phospholipids. This result indicates that the reduction in plasmalogen content is due to a general defect in alkyl ether phospholipid synthesis, rather than to a specific defect in plasmalogen synthesis.

We conclude from the results of Table 1 that at least one enzyme required for plasmalogen synthesis is located in peroxisomes in man and that the absence of peroxisomes results in a reduced activity of this enzyme, either because it is not made or because it is inactive or unstable outside the cell compartment in which it belongs. An intriguing finding is that low but still substantial amounts of plasmalogens are present in erythrocytes of Zellweger patients (Table 1). Erythrocytes lose their peroxisomes early in erythropoiesis¹⁴ and mature erythrocytes are incapable of *de novo* phospholipid synthesis¹⁵. This makes it unlikely that the erythrocyte plasmalogens in Zellweger patients result from a tissue-specific retention of plasmalogen biosynthesis. We rather think that erythrocytes acquire their alkyl ether lipids from dietary sources, as intestines are known to absorb plasmalogen precursors in part without alteration¹⁶. Dietary alkyl ether lipids may also account for the low but significant plasmalogen levels in the parenchymal tissues analysed. If plasmalogens were able to pass through the placenta, the relatively normal duration of gestation of Zellweger patients¹ would be explained.

The only ether lipid with a known physiological function is the platelet-activating factor, an ether lipid containing an acetyl group on the C-2 atom of the glycerol moiety¹⁷. Alkyl and alkenyl ether lipids are, however, ubiquitous membrane constituents throughout nature¹⁸. Their biosynthesis requires several enzymes that are not known to have any other functions in the cell. It is hard to believe that cells would make this investment if ether lipids do not have an essential function in membranes. It is possible, therefore, that the severe reduction in tissue plasmalogen content is sufficient in itself to create the lethal abnormalities present in Zellweger patients. However, there may be other, as yet unknown, metabolic pathways in mammalian peroxisomes that could be the basis of these abnormalities. As argued elsewhere¹¹, we assume that the primary genetic defect in the Zellweger syndrome resides in a gene for a peroxisomal membrane protein or for an enzyme required for import of peroxisomal proteins into the organelle.

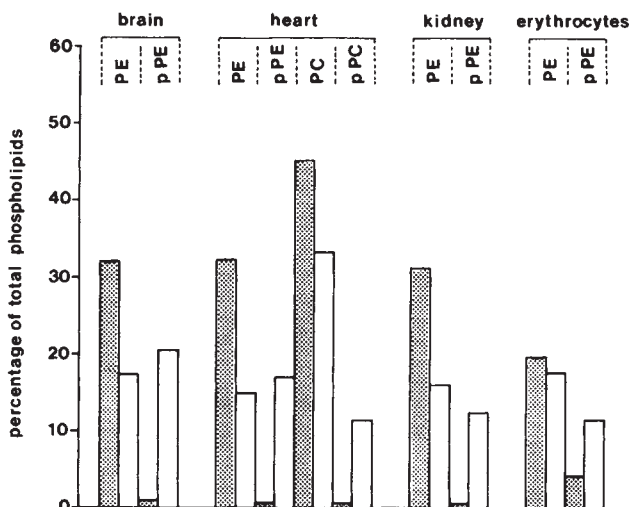


Fig. 1 Representative differences between phospholipids of Zellweger patients (filled bars) and controls (open bars). Data and abbreviations are as in Table 1.

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Table 1 Phospholipid composition of Zellweger patient and control tissues

Tissue	Brain		Kidney		Liver		Muscle		Heart		Erythrocytes	
	ZS (n=2)	Controls (n=2)	ZS (n=4)	Controls (n=2)	ZS (n=4)	Controls (n=2)	ZS (n=2)	Controls (n=2)	ZS (n=2)	Controls (n=2)	ZS (n=3)	Controls (n=10)
% PE	31.6–33.1	17.5–17.6	29.5–32.7	15.5–16.7	31.9–38.4	28.4–29.4	24.6–31.4	11.3–12.3	31.0–33.5	14.9–15.2	17.6–20.2	Mean s.d. 17.6 1.4
% pPE	0.6–1.4	19.3–21.1	0.1–0.9	11.5–13.6	0.3–1.1	2.7–3.5	0.6–0.7	13.8–16.5	0.5–0.8	16.7–17.4	3.8–6.6	11.5 1.0
% PC	43.5–44.4	35.9–43.5	39.6–44.3	40.1–42.1	44.0–48.6	48.4–50.6	49.4–53.0	49.1–51.7	43.5–46.9	33.0–33.7	35.4–39.8	28.6 1.8
% pPC	ND–1.2	ND–0.8	ND–0.4	0.4–1.3	0.2–0.7	ND–0.7	ND–0.8	4.7–5.5	0.1–0.4	11.0–12.0	1.6–2.6	2.3 1.5
[pPE/(PE+pPE)] ×100%	1.8–4.2	52.4–54.5	0.3–2.8	40.8–46.7	0.9–3.3	8.4–10.9	2.2–2.4	53.1–59.3	1.5–2.4	52.4–53.9	13.5–24.6	39.3 1.8

The values are given as a percentage of total phospholipids. Abbreviations: PE, acid-stable phosphatidylethanolamine; pPE, plasmalogen PE; PC, phosphatidylcholine; pPC, plasmalogen PC; ZS, Zellweger syndrome; ND, not detectable. Tissues from five ZS patients (two boys and three girls) were sampled postmortem and stored at -70°C or lower. Erythrocytes of one of these girls and from two additional ZS patients (one girl, one boy) were isolated from heparinized blood. All patients had clinical and biochemical features characteristic of ZS syndrome, including craniofacial dysmorphism, severe hypotonia, liver function disturbances, hyperpepcolic aciduria and abnormal bile acids in urine (see ref. 6). Four patients died before the age of 2 months, one at 11 months. Control tissues were obtained from patients who died because of a disease not related to ZS, such as congenital heart disease and non-ketotic hyperglycaemia. Control erythrocytes were isolated from blood of healthy persons. Phospholipids were extracted from homogenized tissues and erythrocytes according to Bligh and Dyer²⁰. Aliquots of the lipid extract corresponding to about 500 nmol lipid phosphorus were applied to silicagel 60 HR plates and separated into individual components by the method of Horrocks²¹ with slight modifications. The plates were developed in the first direction using chloroform/methanol/water/concentrated ammonia (90:54:5.5:5.5, v/v). After drying the plates for 10 min in a stream of air, they were exposed for 10 min to the fumes of concentrated hydrochloric acid to cleave the vinyl ether linkage of plasmalogens. The plates were then kept for 10 min in a stream of air and finally developed in the second dimension with a solvent mixture consisting of chloroform/methanol/concentrated ammonia (100:50:12, v/v). Phospholipid spots were detected by iodine staining. Quantitation of phospholipids and lysophospholipids originating from plasmalogens was done by measuring phosphorus contents as described by Rouser *et al.*²² Alkaline hydrolysis of isolated phosphatidyl-ethanolamine was carried out as described by Clarke and Dawson²³.

Our results have implications in four areas. First, the determination of plasmalogen synthesis may add to the diagnosis of the Zellweger syndrome and allow the development of a prenatal test for this disease. Second, it is conceivable that exogenous plasmalogens will correct the plasmalogen levels in patients, if given in sufficient amounts. Third, the availability of cells with a defect in ether lipid biosynthesis may provide a useful test system to study the function of plasmalogens in general and of specific ether lipids, like the platelet-activating factor, in particular. And finally, our finding that a major cellular lipid is nearly absent in an inborn error of peroxisome biosynthesis may lead to a revision of the notion that mammalian peroxisomes represent 'the vestige of an ancient organelle'¹⁹, mainly engaged in dispensable pathways of H_2O_2 metabolism.

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Pro- γ -melanocyte-stimulating hormone cleavage in adrenal gland undergoing compensatory growth

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Regulation of the rapid compensatory growth seen in the remaining adrenal gland of rats following unilateral adrenalectomy is poorly understood. The role of adrenocorticotrophic hormone (ACTH) is obscure^{1,2} as immunoneutralization of circulating ACTH does not affect the observed compensatory growth or hyperplasia¹. This finding, together with the fact that mechanical manipulation of one adrenal without extirpation is followed by growth only in the contralateral gland³, has led to the concept of neural regulation of compensatory adrenal growth via a loop from one adrenal through the hypothalamus and back to the contralateral gland which is independent of ACTH secretion³. We recently showed that peptides from the N terminal of ACTH precursor proopiomelanocortin (POC), not containing the γ -melanocyte-stimulating hormone (γ -MSH) sequence, can stimulate adrenal mitogenesis and proposed that normal long-term adrenal growth and proliferation involves post-secretional proteolytic cleavage of pro- γ -MSH [or N-POC(1–74)] to generate the mitogenic factor N-POC(1–48/49) and a C-terminal fragment N-POC(50–74), or rat γ_3 -MSH⁴. We have now investigated this hypothesis further in rats by selectively quenching different regions of circulating POC peptides with specific antisera and observing the effect on the increases in weight, RNA and DNA normally seen in the remaining gland following unilateral adrenalectomy^{1–3}. Our results, reported here, suggest that neurally mediated proteolytic cleavage of the circulating inactive mitogenic precursor pro- γ -MSH at the adrenal gland is the major mechanism of control of compensatory growth.

Dramatic and highly significant increases in adrenal weight, RNA and DNA are seen in the remaining adrenal gland 24 h after unilateral adrenalectomy compared with sham-operated controls (Fig. 1), and provide us with a rapid but objective

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assessment of cellular hypertrophy (RNA) and hyperplasia (DNA). We have used various anti-POC antisera to investigate the mechanism of this effect (Table 1).

In rats treated with normal rabbit serum (NRS), the weight and nucleic acid content of the right adrenal increases significantly by 24 h after excision of the left adrenal (Table 2; weight 32% DNA 71%, RNA 83%). Treatment with anti-ACTH antiserum has no effect on the increase in adrenal weight and DNA (compare with ref. 1) but inhibits the increase in RNA content (18%, $P < 0.05$ compared with 83%, $P < 0.001$, in NRS-treated group). Plasma corticosterone concentrations were significantly less than in the NRS group (53%, $P < 0.001$). Antiserum against the extreme N terminal of POC [anti-POC(1-28)] has no effect on the observed increase in weight, it completely abolishes the increase in DNA content and has little or no effect on the RNA increase (73% compared with 83%). Anti-N-POC(1-76) antiserum has a similar effect on weight and DNA as the anti-N-POC(1-28) antiserum but enhances the RNA increase over the NRS group (138% versus 83%). Neither anti-N-POC antiserum (1-28 or 1-76) has any effect on circulating corticosterone.

These results indicate that N-terminal POC peptides play a major part in compensatory hyperplasia following unilateral adrenalectomy. The inhibition of hyperplasia by both anti-N-POC antisera confirms our previous findings that the mitogenic activity resides in the extreme N terminal of POC⁴. POC is co-secreted with ACTH from the pituitary in an inactive form [as N-POC(1-74) in the rat], and, like ACTH, plasma concentration increases only slightly following extirpation of one adrenal; an independent post-secretional cleavage must therefore be involved. The enhancing effect of the anti-POC(1-76) antiserum on RNA synthesis could be explained by an increased half life of antibody-bound N-POC(1-74) in circulation in which the RNA/steroidogenic-potentiating site (γ_3 -MSH) is not masked. In contrast, with the anti-POC(1-28) antiserum, its more terminally localized antigenic site would not be expected to prevent cleavage of labile sites in the mid-portion of the molecule (positions 49-50).

Whereas the inhibition by anti- γ_3 -MSH antiserum of RNA synthesis was not unexpected⁵, its complete inhibition of DNA synthesis is in contrast with previous *in vitro* results⁴. This

observation does, however, substantiate the proposal that the mitogenic precursor (pro- γ -MSH) has to be cleaved after secretion before it can express its mitogenic activity. Thus when the anti- γ_3 -MSH antiserum masked the RNA-stimulating site it must have simultaneously inhibited the enzymatic cleavage of the mitogenic precursor, preventing expression of its DNA-stimulating activity. The reduced concentration of corticosterone (52% of controls, $P < 0.001$) in this group is consistent with the localization of steroidogenic-potentiating activity of pro- γ -MSH⁶ in the γ_3 -MSH region⁷.

Proopiomelanocortin is synthesized both in the corticotropes of the pars distalis and in the pars intermedia^{8,9}. N-POC(1-74),

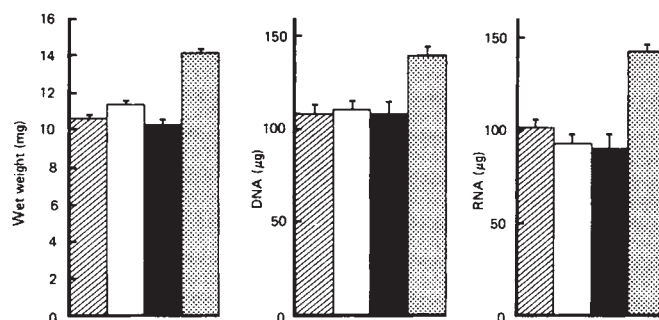


Fig. 1 Five-week-old female Wistar rats were either sham-adrenalectomized (▨), subjected to ether anaesthesia for 15 min (□) or unilaterally adrenalectomized (■). Weight, DNA and RNA content of the right adrenal gland after 24 h were determined as described in Table 2 legend and compared with unoperated controls (■). The right adrenals of the unilaterally adrenalectomized group showed significant differences ($P < 0.001$) in weight, DNA and RNA compared with sham, ether anaesthesia or unoperated control right adrenals.

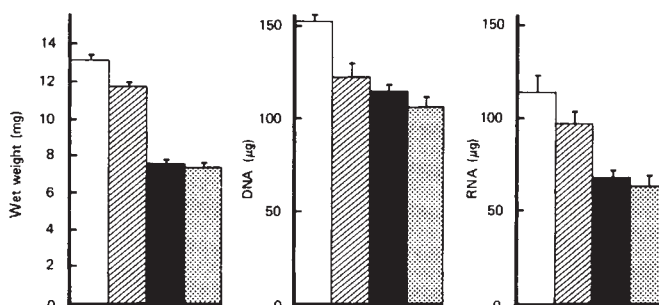


Fig. 2 Effects of dexamethasone (Sigma; ■), bromocriptine (Sandoz; ▨) and dexamethasone plus bromocriptine (□) on compensatory adrenal growth in 5-week-old female Wistar rats compared with controls (□). Six animals were used in each group. Dexamethasone blockade was achieved by adding the compound ($10 \mu\text{g ml}^{-1}$) to drinking water 24 h before unilateral adrenalectomy. Bromocriptine ($0.5 \mu\text{g ml}^{-1}$) was also added to the drinking water 24 h before unilateral adrenalectomy. 24 h after unilateral adrenalectomy all animals were decapitated and blood collected for corticosterone estimation. Right adrenals were removed and processed as described in Table 2 legend. Corticosterone concentrations in the dexamethasone-treated and dexamethasone + bromocriptine-treated groups were significantly depressed (3.43 ± 0.39 and $2.05 \pm 0.39 \mu\text{g per } 100 \text{ ml}$ respectively, compared with control value $27.18 \pm 2.64 \mu\text{g per } 100 \text{ ml}$). Corticosterone levels in the bromocriptine-treated group were slightly depressed compared with control value, 24.16 ± 2.11 . Results are expressed as mean values $\pm$ s.e.m. $n = 8$. In control experiments were sham-operated animals dexamethasone treatment (48 h) showed significant decreases in weight ($7.6 \pm 0.51 \text{ mg}$; untreated $11.17 \pm 1.24 \text{ mg}$) and RNA ($67.62 \pm 3.75 \mu\text{g}$; untreated $109.4 \pm 7.2 \mu\text{g}$) but not in DNA ($76.25 \pm 9.83 \mu\text{g}$; untreated $90.2 \pm 8.35 \mu\text{g}$). The results after bromocriptine treatment (48 h) were not significantly different from untreated animals (weight $12.3 \pm 0.73 \text{ mg}$; DNA $80 \pm 10.4 \mu\text{g}$; RNA $108 \pm 6.1 \mu\text{g}$; $n = 10$. Results are expressed as mean $\pm$ s.e.m.

Table 1 Specificities of the antisera used to investigate peptidergic control of compensatory adrenal growth

ANTISERA	PEPTIDES				
	ACTH	N-POC 1-28	N-POC 1-76	γ_3 -MSH (N-POC 50-74)	β -LPH
ACTH	+	-	-	-	+
N-POC 1-28	-	+	+	-	-
N-POC 1-76	-	+	+	-	-
γ_3 -MSH (N-POC 50-74)	-	-	+	+	-

Characteristics of the peptides ACTH, β -LPH, N-POC(1-76) in the rat it is N-POC(1-74)²³, being two residues shorter than in man and N-POC(1-28) have been described previously^{4,9,21}. The anti-N-POC(1-76) antiserum used was similar to that described previously¹⁰, except that it partially cross-reacted with N-POC(1-28). The anti- γ_3 -MSH and anti-N-POC(1-28) antisera were raised in rabbits by immunization with the respective peptides coupled to bovine thyroglobulin by glutaraldehyde. Synthetic rat γ_3 -MSH was a gift from Dr Nicholas Ling. Binding was assessed by the binding of iodinated peptides to each antiserum, except in the case of N-POC(1-28) which has no tyrosine, but as 70% of radioiodinated N-POC(1-76) bound to the N-POC(1-28) antiserum and was equipotent with N-POC(1-28) in the radioimmunoassay it could be considered to have full cross-reaction.

Table 2 Effect of antisera on adrenal weight and nucleic acid concentration following unilateral adrenalectomy

Antiserum	No. of rats	Corticosterone (μg per 100 ml)	Weight (mg)	Left adrenal DNA (μg)	RNA (μg)	Weight (mg)	Right adrenal DNA (μg)	RNA (μg)
Control (NRS)	10	33.27 $\pm$ 3.02	9.0 $\pm$ 0.47	108.2 $\pm$ 5.2	88.8 $\pm$ 7.2	11.91 $\pm$ 0.61*	185.57 $\pm$ 6.18*	161.9 $\pm$ 8.8*
Anti-N-POC (51-74)	8	15.97 $\pm$ 2.48	8.61 $\pm$ 0.81	90.75 $\pm$ 6.83	94.12 $\pm$ 8.82	9.05 $\pm$ 0.37†	85.25 $\pm$ 7.41†	112.25 $\pm$ 9.15‡
Anti-N-POC (1-28)	8	29.06 $\pm$ 3.02	9.22 $\pm$ 0.39	102.75 $\pm$ 8.01	113.12 $\pm$ 2.64	11.24 $\pm$ 0.79§	108.07 $\pm$ 7.39†	195.25 $\pm$ 12.69*
Anti-N-POC (1-76)	8	29.39 $\pm$ 3.49	9.93 $\pm$ 0.49	103.75 $\pm$ 6.88	112.87 $\pm$ 3.59	14.52 $\pm$ 0.72*	101.25 $\pm$ 6.75†	268.25 $\pm$ 11.68*¶
Anti-ACTH (4-10)	7	13.87 $\pm$ 2.34	11.12 $\pm$ 0.59	80.71 $\pm$ 7.0	91.5 $\pm$ 4.5	15.01 $\pm$ 1.11*	130.0 $\pm$ 8.59*	101.42 $\pm$ 4.0‡

All values are mean $\pm$ s.e.m. The significance of the difference between left and right adrenal was determined by paired *t*-test, and intergroup differences compared by analysis of variance. There was no significant difference between the groups with respect to whole body weight. RNA and DNA contents were calculated as total content (μg) per adrenal gland. Five-week-old female Wistar rats were injected [200 μl intraperitoneally (i.p.) and 200 μl subcutaneously (s.c.)] with neat antiserum 2 h before unilateral adrenalectomy. Rats were anaesthetized with ether and the left adrenal gland removed through a retroperitoneal incision. All skin incisions were clipped (Michael Suture Clips, Downs Surgical, London) and the rats returned to their cages. The glands were trimmed of fat, membranes weighed to the nearest 0.01 mg and placed on 0.9% saline at 4 °C until nucleic acid extraction. RNA and DNA contents were determined by the method of Schneider²⁵, which is based on the differential measurement of pentose with orcinol²⁶ (Sigma) and desoxypentose with diphenylamine²⁷ (BDH). Calf thymus DNA (Sigma) and D-ribose (Sigma) were used as standards. 24 h after unilateral adrenalectomy the rats were killed by decapitation and blood collected for corticosterone estimation. Right adrenals were removed and processed in the same manner as the left adrenals. In control sham-operated animals, attempts to show any significant difference in any of the three parameters 24 h after injection of the various antisera proved negative. Treatment of animals for 72 h with four-times concentrated antisera administered by implanted Alzet osmotic minipumps (Model 2001, Scientific Marketing, London) did not result in any significant difference between groups either.

* Significantly greater than left adrenal ($P < 0.001$). † Not significant. ‡ Significantly greater than left adrenal ($P < 0.05$). § Significantly greater than left adrenal ($P < 0.01$). || Significantly greater than control right adrenal ($P < 0.05$). ¶ Significantly greater than control right adrenal ($P < 0.001$).

Table 3 Effect of left adrenal manipulation

Treatment	Weight (mg)	DNA (μg)	RNA (μg)
Rat anti- γ_3 -MSH antiserum			
Left adrenal	10.13 $\pm$ 83	91.5 $\pm$ 5.94	75.75 $\pm$ 5.68
Right adrenal	9.89 $\pm$ 0.69*	85.0 $\pm$ 7.45*	82.0 $\pm$ 6.33*
NRS			
Left adrenal	8.60 $\pm$ 0.61	72.0 $\pm$ 3.0	69.3 $\pm$ 2.95
Right adrenal	10.22 $\pm$ 0.68†	99.0 $\pm$ 3.28†	94.87 $\pm$ 3.23†

Left adrenal manipulation was carried out 2 h after injection of either NRS or rat anti- γ_3 -MSH [N-POC(51-74)] antiserum (200 μl i.p. and 200 μl s.c.) into 5-week-old female Wistar rats. Adrenal manipulation was performed as previously described³ by clamping the pedicle for a few seconds until the gland blanched peripherally and flushed centrally. All three parameters were measured as described in Fig. 2 legend. Values are mean $\pm$ s.e.m. $n = 8$.

* Not significant.

† Significantly different from left adrenal ($P < 0.001$).

ACTH, β -lipotropin (β -LPH) and some β -endorphin are the primary secretory products of the pars distalis¹⁰⁻¹², but considerable further processing takes place in the pars intermedia giving rise to a variety of smaller peptides depending on the species¹³⁻¹⁵. N-POC(1-74), unlike the rest of POC, is only partially processed (~30%) in the rat pars intermedia¹⁶, so the mature adrenal mitogenic factor, N-POC(1-48/9) and γ_3 -MSH are secreted.

To determine the source of peptides involved in compensatory adrenal growth, dexamethasone was used to inhibit selectively hypothalamic corticotrope activity and bromocriptine to inhibit secretion from the pars intermedia^{17,18}. We found that whereas dexamethasone completely prevented the normal increase in weight, DNA and RNA seen in the remaining adrenal gland after unilateral adrenalectomy (Fig. 2), with bromocriptine the normal increase in weight and RNA was observed but only a small, though significant (17%, $P < 0.02$), increase in DNA content compared with control (52%). This suggests that the pars intermedia N-POC peptides are mitogenic in the adrenal (hence the reduced DNA increase), although it could be accounted for by other bromocriptine-mediated effects on the adrenal. The lack of difference in RNA increase in the bromocriptine group compared with control suggests that the secretion of intact ACTH from the pars distalis is permissive in the γ_3 -MSH-induced hypertrophy. This is consistent with the similar inhibitory effects of anti-ACTH and anti- γ_3 -MSH antisera on RNA (Table 2). These observations suggest that the pars distalis corticotrope is the primary source of peptides involved in adrenal hypertrophy and hyperplasia.

That spinal cord hemisection in the thoracic region inhibits compensatory adrenal growth when the contralateral adrenal (but not the ipsilateral) is removed, and pretreatment of the

left adrenal with an anaesthetic (lidocaine) before removal inhibits the subsequent compensatory adrenal growth, have led Dallman *et al.* to conclude that both afferent and efferent neural elements mediate adrenal growth after unilateral adrenalectomy¹⁹. In our experiments we have been able to imitate the effects of ablation of these neural elements by pretreatment with certain antisera. Moreover, the findings that γ_3 -MSH does not possess mitogenic activity and that the N-terminal region of pro- γ -MSH has to be cleaved before it can express its mitogenic activity suggest that while the anti- γ_3 -MSH antiserum quenches the RNA-stimulating activity of N-POC(1-74) (the γ_3 -MSH region), it also prevents proteolytic cleavage, probably at the double basic residues at positions 48-49. Neural regulation is further suggested by the fact that compensatory growth is still seen only in the contralateral gland after temporary occlusion of the blood flow to one adrenal and is not accompanied by any increase in plasma ACTH concentrations compared with sham controls³. We were able to abolish completely the increases in weight, RNA and DNA that normally follow adrenal manipulation by pretreatment with the anti- γ_3 -MSH antiserum (Table 3). The increase in weight, RNA and DNA in the NRS group is not as dramatic as that following unilateral adrenalectomy, but still highly significant.

As we can inhibit what has been regarded as neurally mediated stimulation of adrenal growth with an antiserum which seems to prevent enzyme-induced expression of the mitogenic activity of pro- γ -MSH, it seems that adrenal hypertrophy and hyperplasia are mediated by neural activation of a proteolytic enzyme, independently in each adrenal gland, which cleaves pro- γ -MSH (N-POC(1-74)) to give rise locally to N-POC(1-48/49) and γ_3 -MSH (N-POC(50-74)). N-POC(1-48/49) stimulates DNA synthesis and mitogenesis and γ_3 -MSH stimulates RNA synthesis and hypertrophy. ACTH seems to have a permissive role in the latter case. Hypersecretion of pro- γ -MSH and ACTH from the corticotropes thus would only lead to an increase in RNA synthesis and hypertrophy. DNA synthesis (and subsequent hyperplasia) would only occur following neural activation of the proteolytic enzyme responsible for the cleavage of pro- γ -MSH. The main source of peptides involved in this action seems to be the corticotropes of the pars distalis and is in agreement with the lack of adrenal effects following removal of intermedia tissue²⁰.

We propose that N-POC(1-48/49) be named adrenal mitogenic hormone, γ_3 -MSH or N-POC(50-74) adrenal hypertrophic hormone and pro- γ -MSH or N-POC(1-74) (in the rat) adrenal growth factor. Independent post-secretional modification/activation of inactive co-secreted hormone precursors at separate target sites may provide a more refined and subtle control mechanism than the co-secretion of many active hormones under a single release stimulus.

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Linkage between surface immunoglobulin and cytoskeleton of B lymphocytes may involve Gc protein

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The membrane immunoglobulin (MIg) of B lymphocytes is thought to have an important role in antigen recognition and cellular activation^{1,2}. In common with many membrane glycoproteins, MIg moves extensively in the lipid bilayer, and after binding of specific antisera displays lateral mobility with patch and cap formation^{3,4}. This phenomenon appears to involve the cytoskeleton, particularly the actin that is present in the cell membrane of B lymphocytes⁵ and aggregates beneath capped immunoglobulin⁶. Recently, it has been reported that the isolation of MIg results in co-purification of actin and an unknown protein of molecular weight (MW) ~56,000 (refs 7, 8). We now demonstrate that this component displays physicochemical and immunological properties indistinguishable from those of Gc (group-specific component)⁹. In addition, evidence is presented which suggests that this vitamin D₃-binding protein is involved in the linkage between MIg and actin, and may therefore be important in signal transduction.

The possible involvement of Gc protein in this process was suggested by recent reports of high-affinity interactions of Gc with actin^{10,11}, coupled with the knowledge that the MW of this protein (56,000)⁹ is identical to that of a major membrane species associated with MIg^{7,8}. To test this hypothesis, model studies of interactions between Gc, immunoglobulin and actin were performed with these purified components^{12,13}. Serum immunoglobulin was immobilized on an affinity column consisting of glutaraldehyde-activated AH-Sepharose 4B¹⁴ coupled with F(ab')₂ fragments of a goat antiserum to human IgG F(ab')₂ which bound all classes of immunoglobulin. Subsequently, Gc and/or actin were applied and binding was assessed by monitor-

ing both unbound protein and the bound fraction eluted with 3 M ammonium thiocyanate¹⁴. It was clearly shown that purified Gc, and preformed equimolar complexes of Gc and actin¹², were bound whereas actin alone was not (Fig. 1). In contrast, when Gc or Gc-actin complexes were applied without prior attachment of immunoglobulin, no binding was detected (Fig. 1), thus effectively excluding unwanted binding of Gc or actin directly to immunoadsorbent gel. These studies indicate, therefore, that Gc can bind both actin and immunoglobulin contemporaneously.

Similar studies were then performed *in situ* on normal human peripheral blood lymphocytes (PBL) depleted of monocytes^{7,8}. After solubilization in non-ionic detergent^{7,8}, soluble supernatants obtained by ultracentrifugation were subjected to chromatography on the same anti-immunoglobulin immunoabsorbent column¹⁴. Specifically bound protein was then eluted with thiocyanate¹⁴ and examined both physicochemically and immunochemically. Analysis by SDS-polyacrylamide gel electrophoresis (PAGE)¹⁵ in reducing conditions, together with silver staining¹⁶ (Fig. 2), demonstrated immunoglobulin and at least two additional species of MW 56,000 and 42,000, which co-migrated with purified standards of serum Gc and skeletal muscle G-actin¹², respectively. SDS-PAGE gels were also examined immunochemically after electroblotting¹⁷ with antisera specific (see Fig. 1) for immunoglobulin, Gc and actin. This confirmed that the 56,000- and 42,000-MW species that eluted with immunoglobulin were immunologically identical to Gc and actin, respectively (Fig. 2). Control experiments in the presence of detergent again showed that neither purified Gc protein nor complexes of Gc and G-actin bound directly to the column.

These findings provided further evidence for the formation of a two-way complex between Gc and both immunoglobulin and actin. However, they did not indicate whether the relevant interactions between these components occurred *in vivo* on the surface of B lymphocytes or *in vitro* after release from the membrane. Thus, detergent treatment of whole cells would be expected to solubilize actin and any Gc protein present in the cytoplasm^{10,11}, in addition to material on the membrane. However, when the surface membrane of intact cells was radioiodinated⁵ before solubilization, both the 42,000-MW actin and

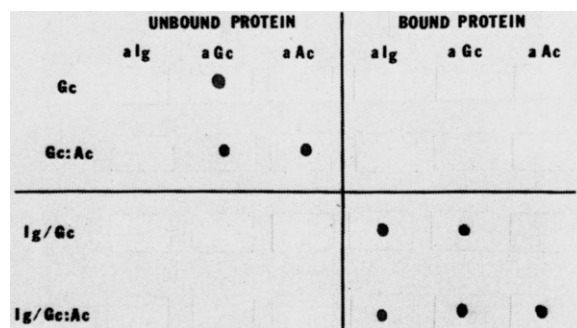


Fig. 1 Formation of complexes between purified immunoglobulin, Gc protein and actin (Ac)^{12,13}. Immunoglobulin was first immobilized on an anti-immunoglobulin column as described in the text, and Gc and/or actin were offered for binding. The unbound fraction, and bound protein eluted with 3 M ammonium thiocyanate, were examined for the different components added by a sensitive immunological method. In this procedure, which is a modification of previously described blotting techniques¹⁷, protein is spotted directly onto nitrocellulose sheets and analysed with rabbit antisera specific for the test proteins (aIg, aGc and aAc), followed by peroxidase-labelled goat antiserum to rabbit immunoglobulin and development of the reaction with diaminobenzidine¹⁷. This method is sensitive, and detects less than 1 ng protein¹⁷. The fractions were analysed for immunoglobulin, and actin, using rabbit antisera, each of which was highly specific for homologous antigen and showed negligible cross-reaction with the other two proteins. Note that neither Gc nor equimolar complexes of Gc-actin, bound to the column in the absence of bound immunoglobulin, whereas both these samples clearly bound in the presence of immunoglobulin.

Fig. 2 Detection of complex formation on the membrane of peripheral blood lymphocytes (PBL). Cells were obtained by Ficoll-Isopaque centrifugation of heparinized blood from normal volunteers; monocytes were depleted by adherence to plastic Petri dishes, and the final preparations contained >95% lymphocytes. Cells (10^8) were exposed to 1% Nonidet P-40 at 4 °C for 30 min in 10 mM Tris-HCl pH 7.5, 50 mM KCl, in the presence of 1 mM phenylmethylsulphonyl fluoride⁷, and solubilized material was recovered by centrifugation (100,000g for 30 min at 4 °C). Immunoglobulin and associated protein was isolated by affinity chromatography on a column prepared by coupling of a polyvalent antiserum to human immunoglobulin (goat anti-IgG F(ab')₂) to glutaraldehyde-activated AH-Sepharose 4B¹⁴. Bound material was eluted with 3 M ammonium thiocyanate¹⁴, dialysed immediately against 62.5 mM Tris-glycine buffer¹⁵ containing 2.3% SDS and 10% glycerol, and concentrated before SDS-PAGE, which was performed with and without reduction in the presence of 10% mercaptoethanol¹⁵. The protein bands obtained were then either directly stained with silver¹⁶, or transferred to nitrocellulose paper by electroblotting¹⁷ for further immunological analysis with the same antisera to immunoglobulin, Gc and actin used for Fig. 1. Each gel included molecular weight standards, and standards of purified serum Gc protein, rabbit skeletal muscle actin, and human immunoglobulin. Lane a, unbound fraction from immunoabsorbent column; b, material bound to anti-immunoglobulin column, both reduced and stained with silver. Lanes c-e, immunoblots of bound material shown in b after reaction with antisera to immunoglobulin (c), Gc protein (d) and actin (e). Note co-isolation of Gc and actin with immunoglobulin.

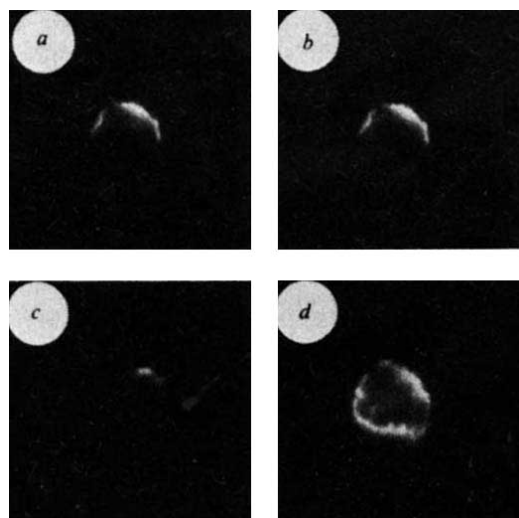
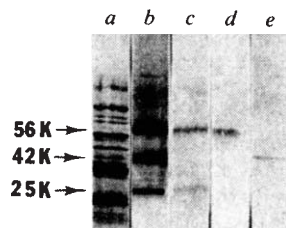


Fig. 3 Co-capping of membrane immunoglobulin (MIg) and Gc protein. PBL prepared as described in Fig. 2 legend were examined concurrently for membrane mobility of surface MIg and Gc by two-colour fluorescence⁶. Cells were reacted with a fluorescein-labelled polyvalent goat antiserum to human immunoglobulin at 4 °C, then warmed to 37 °C for 30 min to induce capping. Cells were then incubated at 4 °C with a rabbit antiserum to human Gc, followed by tetramethylrhodamine-labelled sheep antiserum to rabbit immunoglobulin. Subsequent examination for green (a) and red (b) fluorescence revealed that B lymphocytes were positive for Gc, and showed exact correspondence between red and green patches and caps. Control experiments in which phosphate-buffered saline or non-immune rabbit or goat serum was substituted for the rabbit anti-Gc and goat anti-immunoglobulin antisera, respectively, were uniformly negative. Note that after similar capping of immunoglobulin with anti-immunoglobulin (c), incubation of cells with tetramethylrhodamine-labelled antiserum to framework determinants of DR antigen¹⁹, failed to demonstrate any evidence of co-capping of DR antigens (d).

the 56,000-MW Gc bands were radiolabelled, indicating origin at least in part from the membrane. Viable cells were then reacted with a fluorescein-labelled antiserum to human immunoglobulin to induce lateral mobility¹⁸ of the putative membrane complex with Gc and actin. Redistribution after warming the cells to 37 °C was studied by concurrent examination with a tetramethylrhodamine-labelled anti-Gc antiserum⁶. The typical patching and capping of MIg evident by green fluorescence (Fig. 3a) was exactly paralleled by co-capping of Gc protein in red (Fig. 3b). Capping of Gc could not, however, be induced directly with anti-Gc, and previous reaction of cells with anti-Gc did not prevent capping of both immunoglobulin and Gc upon subsequent reaction with anti-immunoglobulin. In contrast, studies of another major membrane protein expressed by B lymphocytes (DR), performed with a tetramethylrhodamine-labelled antiserum to framework determinants of DR antigens¹⁹, showed no evidence of co-mobility on capping of immunoglobulin with anti-immunoglobulin (Fig. 3c,d).

These observations are consistent with the concept that immunoglobulin molecules on the surface membrane of B lymphocytes can become associated spatially with a protein indistinguishable from Gc, which is in turn linked to actin^{10,11}. It is unknown whether the Gc detected is an integral membrane protein or is bound from the large pool available in serum. Although the only previous study in lymphocytes of which we are aware reports exogenous binding²⁰, it should be stressed that membrane Gc behaves in many respects as an integral membrane protein, being unaffected by washing with 3 M KCl or ammonium thiocyanate and requiring detergent solubilization for its release. Nevertheless, although its origin remains to be determined, it appears that Gc protein can bind both immunoglobulin and actin in the membrane and could therefore provide a mechanism for linkage of surface immunoglobulin to the cytoskeleton. This does not imply that Gc and immunoglobulin are necessarily permanently attached on the membrane. Spatial association could be initiated *de novo* by configurational changes in MIg, such as might be induced by binding of specific antibody or homologous antigen, and Gc might then contribute to the signal transduction which culminates in cellular activation⁴. The interactions observed between Gc and serum immunoglobulin bound to the immunoabsorbent column are consistent with this concept. Finally, as actin and vitamin D-binding protein identical to Gc have been found in all nucleated cells examined to date²¹, and as many other surface components display membrane mobility, it will therefore be of interest to explore the possibility that Gc contributes to linkage of other membrane glycoproteins to the cytoskeleton.

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Direct enzyme transfer from lymphocytes is specific

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Lymphocytes are known to interact directly with other cells *in vivo*^{1,2} and *in vitro*³⁻⁵, and have recently been shown to transfer the lysosomal enzyme, β -glucuronidase, to fibroblasts from patients with an inherited deficiency of the enzyme⁶. This process requires cell-cell contact, is unaffected by inhibitors of 'classical' receptor-mediated endocytosis and is abolished by inhibitors of protein synthesis⁷. Although it is not yet known to what extent the transfer of enzymes by direct cellular interaction is a general phenomenon, a similar mechanism could possibly be involved in the transfer of other lysosomal enzymes *in vivo*⁸⁻¹⁰ and in the exchange of protein *in vitro*¹¹. We show here that the direct transfer of enzymes from lymphocytes to fibroblasts is restricted to only certain lysosomal enzymes.

Concanavalin A (Con A)-transformed mouse spleen lymphocytes were co-cultured for 24 h, as previously described⁶, with cells from several human fibroblast lines each genetically deficient in a different enzyme. The lymphocytes were removed and enzyme activities were measured in extracts of the isolated fibroblasts⁷. The following lysosomal enzymes, α -L-iduronidase (EC 3.2.1.76), arylsulphatase (EC 3.1.6.1) and β -hexosaminidase (EC 3.2.1.30), increased in activity after co-culture by 290%, 381% and 1,190% respectively, thereby reaching 33%, 42% and 7.4% of the activities present in extracts of normal human fibroblasts (Table 1). The levels of both α -L-iduronidase and arylsulphatase acquired during co-culture were thus comparable with those in fibroblasts from obligate heterozygotes who are clinically normal¹², and even the activity acquired by β -hexosaminidase-deficient fibroblasts would probably be sufficient to correct the metabolic defect¹³⁻¹⁵. By contrast, a fourth lysosomal enzyme, β -glucosidase, was not transferred to the deficient fibroblasts, whose activity remained unchanged after co-culture with lymphocytes (Table 1).

As had previously been shown with β -glucuronidase^{6,7}, the acquisition of β -hexosaminidase activity by deficient fibroblasts also required direct cell-cell contact with lymphocytes. Thus, when the lymphocytes were placed inside a Millipore membrane-sealed chamber and thereby separated from the fibroblasts during co-culture, their β -hexosaminidase activity remained at a low level (16.5 nmol per h per 10^6 cells) comparable with that of fibroblasts cultured alone (12.1 nmol per h per 10^6 cells), whereas when the two types of cell were cultured together in direct contact the fibroblasts acquired high activity (156.4 nmol per h per 10^6 cells). Previous studies of β -glucuronidase transfer have shown that lack of enzyme acquisition by deficient fibroblasts when they are separated from the lymphocytes during co-culture is not due to adsorption of lymphocyte enzyme to the Millipore membrane⁶.

The acquisition of β -hexosaminidase activity by deficient fibroblasts did not occur by receptor-mediated endocytosis of enzyme secreted by the lymphocytes. Lymphocytes cultured alone for 24 h secreted less than 0.5% of their intracellular β -hexosaminidase activity, and deficient fibroblasts cultured in the medium in which lymphocytes had previously been

incubated had the same low activity as those cultured in fresh medium (27.8 nmol per h per 10^6 cells). Even when cultured in the presence of extracts of lymphocytes which had β -hexosaminidase activity of 77 nmol per h, the enzyme activity in the fibroblasts did not increase significantly (37.9 nmol per h per 10^6 cells) compared with the enhanced activity after co-culture with intact lymphocytes (156.4 nmol per h per 10^6 cells). Moreover, the acquisition of β -hexosaminidase did not occur by the mannose 6-phosphate receptor system which is involved in the specific endocytosis of several lysosomal enzymes¹⁶⁻¹⁹. Thus, when 10 mM mannose 6-phosphate, a potent

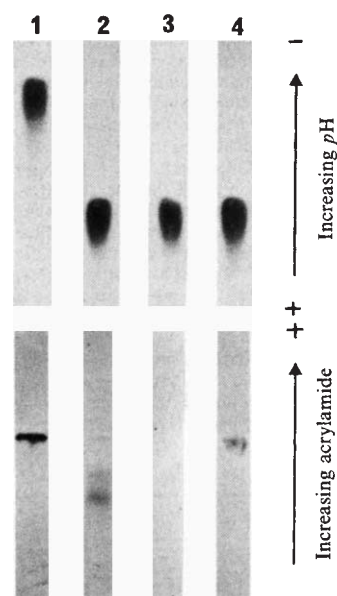


Fig. 1 Effect of co-culture with mouse lymphocytes on glucose 6-phosphate isomerase (a) and β -glucuronidase (b) activities in β -glucuronidase-deficient human fibroblasts. Band 1, mouse lymphocytes, 10^7 cells; band 2, normal human fibroblasts, 10^6 cells; band 3, β -glucuronidase-deficient human fibroblasts, 10^6 cells; band 4, β -glucuronidase-deficient human fibroblasts, 10^6 cells, co-cultured with mouse lymphocytes.

Methods: Fibroblasts obtained from a patient with mucopolysaccharidosis type VII (β -glucuronidase deficiency) were co-cultured with normal murine lymphocytes for 24 h, and the adherent cells were depleted of contaminating lymphocytes as previously described³⁵. a, Glucose 6-phosphate isomerases of human and mouse origin were distinguished by electrofocusing in the following way. The washed cell pellet was mounted in Tissue Tek OCT (Lamb) on a cork disk and frozen in isopentane to -165°C . Two 5- μm -thick sections were cut and collected on filter paper 1×4 mm, and placed onto the surface of a polyacrylamide gel 0.8-mm thick. This had been prepared previously by mixing 10 ml of 29.1% acrylamide, 10 ml of 0.9% bisacrylamide, 10 ml of 2.5 mM dithiothreitol, 7 ml of glycerol, 2 ml of LKB ampholyte, pH 9-11, and 1 ml of ampholyte, pH 8-9.5. After removal of gas under vacuum for 10 min, 2 ml of 1% ammonium persulphate were added and the gel polymerized in a 'casting' tray. The electrofocusing wick (Bio-Rad) at the anodal end was soaked in 2% ampholyte, pH 6-8, and at the cathodal end in 1 M NaOH. The enzymes absorbed from the cryostat sections were then electrofocused to their isoelectric points for 2.5 h at 10°C at 1,500 V. Glucose 6-phosphate isomerase activity in the 10×25 cm polyacrylamide gel was visualized by means of an overlay with a second gel made by mixing the following components at 44°C : 9 ml of 2% agar, 9 ml of 0.36 M Tris-HCl, pH 8.0, 3 ml of 0.25 M MgCl_2 , 3 ml of 0.1 M fructose 6-phosphate, 3 ml of 6 mM NADP, 3 ml of 2 mM nitroblue tetrazolium, 3 ml of 0.8 mM phenazine methosulphate and 30 μl of glucose-6-phosphate dehydrogenase, 1 mg per ml (Boehringer, Grade 1). The agar overlay was gently poured onto the surface of the polyacrylamide gel, over which it formed a thin solidified film at 20°C . This was incubated for 10 min at 37°C and photographed. b, β -Glucuronidases of human and mouse origin were distinguished by polyacrylamide gradient gel electrophoresis in the following way. Duplicate washed pellets of cells were extracted and β -glucuronidase purified by DEAE-cellulose chromatography as previously described³⁵. Sucrose was added to a final concentration of approximately 40%, and 25 μl aliquots of the extract, containing 1-3 nmol per h of β -glucuronidase, were placed onto 2.5-27% concave gradient Gradiopore gels (Universal Scientific). These were subjected to electrophoresis for 24 h at 4°C at 125 V, using a buffer containing 88.7 mM Tris-HCl, 83 mM borate and 2.5 mM EDTA, pH 8.3. β -Glucuronidase activity was visualized by a simultaneous coupling procedure using naphthol-AS-BI- β -glucuronide and pararosaniline at pH 5.0 (refs 38, 39). The gels were incubated for 18 h at 37°C , washed with several changes of 7.5% acetic acid and photographed.

Table 1 Effect of co-culture with lymphocytes on lysosomal enzyme levels in deficient fibroblasts

Enzyme deficiency	No. of experiments	Lymphocytes alone	Average enzyme activity (nmol per h per 10 ⁶ cells)			Average transfer index
			Normal fibroblasts	Deficient fibroblasts	Co-cultured fibroblasts	
α -L-Iduronidase	4	1.31 (0.44–1.81)	13.5 (8.2–22.2)	1.22 (0.91–1.61)	4.46 (3.69–5.50)	290 (129–385)
Arylsulphatase	3	0.93 (0.80–1.06)	46.3 (34.5–55.1)	4.82 (1.40–8.70)	19.4 (4.4–35.1)	281 (211–328)
β -Hexosaminidase	4	65.4 (48.8–88.2)	2,936.6 (2,021.8–4,950.0)	15.6 (9.7–27.8)	217.7 (74.6–493.1)	1,190 (490–1,670)
β -Glucosidase	4	17.4 (8.2–28.9)	92.2 (46.4–176.3)	7.5 (3.5–11.2)	7.1 (3.3–11.3)	1 (–33–42)

Monolayers of enzyme-deficient fibroblasts and Con A-transformed mouse spleen lymphocytes were prepared as previously described⁶. Co-cultures were initiated by adding 10⁸ lymphocytes in 10 ml of minimal essential medium to fibroblasts grown to stationary phase in 75-cm² culture flasks (~10⁶ cells). After 24 h the co-cultured fibroblasts were purified by centrifugation through a Percoll gradient³⁵. They were washed with phosphate-buffered saline (PBS) and the numbers of fibroblasts and remaining lymphocytes determined by direct counting using a haemocytometer. Suspensions of these, and of the other cells as shown, were centrifuged and the pellets were subjected to six cycles of freezing and thawing in 0.5 ml of 0.1% Triton X-100 in water. After centrifugation at 400g for 10 min, duplicate aliquots of the cell-free supernatants were assayed for lysosomal enzyme activities as described below. α -L-Iduronidase: fibroblasts deficient in α -L-iduronidase were obtained from a patient with Hurler's syndrome. The incubation mixture contained 150 μ l of the enzyme extract, 50 μ l of 2.5 mM 4-methylumbelliferyl- α -L-iduronide in 0.2 M formate buffer, pH 2.8, and 50 μ l of 0.1 M boiled saccharate. β -Hexosaminidase: fibroblasts deficient in β -hexosaminidase were obtained from a patient with Sandhoff's disease. The incubation mixture contained 50 μ l of the enzyme extract and 200 μ l of 2 mM 4-methylumbelliferyl-N-acetyl- β -D-glucosaminide in 0.1 M citrate buffer, pH 4.5. Arylsulphatase: fibroblasts deficient in arylsulphatase were obtained from a patient with multiple sulphatidosis. The incubation mixture contained 50 μ l of the enzyme extract and 200 μ l of 7.5 mM 4-methyl-umbelliferyl-sulphate in 0.1 M acetate buffer, pH 5.7. β -Glucosidase: Fibroblasts deficient in β -glucosidase were obtained from a patient with Gaucher's disease. The incubation mixture contained 20 μ l of the enzyme extract, 100 μ l of 10 mM 4-methylumbelliferyl- β -D-glucoside in 50 mM taurocholate, and 50 μ l of 0.45 M phosphate-citrate buffer, pH 5.5. All incubations were carried out for 1 h at 37 °C, after which 1 ml of 0.4 M glycine buffer, pH 10.4, was added and the fluorescence at 448 nm measured in a Locarte fluorimeter (excitation 368 nm). Enzyme activities are expressed as nmol per h per 10⁶ cells used to prepare the cell-free enzyme extract. The arithmetic means of the individual experiments are shown, and numbers in parentheses represent the range of activities between individual experiments. The activity contributed by the presence of contaminating lymphocytes (0.3% of the number added initially) was subtracted from the total activity of the whole extract, as previously described⁷. Enzyme activity transferred during co-culture, thus determined, was previously shown in the case of β -glucuronidase to be comparable with the average activity of individual cells measured by a single-cell cytochemical procedure⁶. Transfer index is per cent change in enzyme activity in deficient fibroblasts after co-culture compared with the enzyme level in fibroblasts cultured alone. Arithmetic means of the transfer index of individual experiments are shown, and numbers in parentheses represent the range of transfer indices between individual experiments. Lack of any enzyme transfer is indicated by an index of zero.

Table 2 Effect of co-culture with lymphocytes on the activities of non-lysosomal enzymes in deficient fibroblasts

Enzyme deficiency	No. of experiments	Lymphocytes alone	Enzyme activity (nmol per h per 10 ⁶ cells)			Transfer index
			Normal fibroblasts	Deficient fibroblasts	Co-cultured fibroblasts	
Galactose 1-phosphate uridylyltransferase	1	3.60	85.9	0	0	0
	2	1.82	15.1	0.81	0.87	7
Hypoxanthine-guanine phosphoribosyltransferase	1	2.20	92.0	0	0	0
	2	1.26	87.0	0	0	0
	3	1.54	34.1	0	0	0
Glucose 6-phosphate dehydrogenase	1	—	203 (14)	55 (5)	67 (7)	22
	2	—	444 (39)	132 (9)	93 (9)	–30
	3	—	237 (19)	125 (8)	121 (9)	–3
	4	—	334 (24)	142 (8)	209 (11)	47
Average		—	305	114	123	9

Fibroblasts deficient in non-lysosomal enzymes were co-cultured for 24 h with lymphocytes and separated from them on Percoll gradients, as described in Table 1 legend. Cell-free extracts were prepared from these and from suspensions of other cells shown, and were assayed as described below. Fibroblasts deficient in galactose 1-phosphate uridylyltransferase were obtained from a patient with galactosaemia. Enzyme activity was measured by a procedure in which the formation of UDP-¹⁴C-galactose, from ¹⁴C-galactose-1-phosphate and uridine diphosphate glucose, was assessed by binding to DEAE-cellulose paper after degradation of unconverted ¹⁴C-galactose-1-phosphate by alkaline phosphatase³⁵. Fibroblasts deficient in hypoxanthine-guanine phosphoribosyltransferase were obtained from a patient with Lesch-Nyhan disease. Enzyme activity was measured by a procedure in which the formation of ¹⁴C-IMP, from ¹⁴C-hypoxanthine and 5-phosphoribosyl-1-pyrophosphate, was assessed by binding to DEAE-cellulose paper³⁷. Enzyme activities are expressed as nmol of product produced per h per 10⁶ cells used to prepare the enzyme extract. Changes in glucose 6-phosphate dehydrogenase activity were measured using a cytochemical procedure. Cell suspensions were centrifuged and resuspended in 1 ml of freshly prepared 0.3% neotetrazolium chloride in 0.1 M Tris-HCl, pH 7.4, containing 1.5 mg glucose-6-phosphate, 2.5 mg NADP, 10 μ mol sodium cyanide and 0.2 mg phenazine methosulphate. After incubation for 1 h at 30 °C, the cells were centrifuged, washed and resuspended in PBS. Aliquots were air-dried on slides, then fixed with methanol, washed with water, re-dried and mounted in Farrants medium. The absorption densities of the cells at 585 nm were determined on a Vickers microdensitometer M-85 using a B5 mask. Dehydrogenase activities are expressed as the arithmetic means ($\pm$ s.e.) of the densities of 30–100 individual fibroblasts. Co-cultured fibroblasts were readily distinguishable from contaminating lymphocytes by their shape and larger size.

inhibitor of this uptake system, was added to the co-cultures, the β -hexosaminidase activity of the deficient fibroblasts still increased (to 164.1 nmol per h per 10⁶ cells).

To eliminate the possibility that contact with lymphocytes induces the synthesis of active enzyme by the fibroblasts, lymphoblastoid cells deficient in α -L-iduronidase (0.102 nmol per h per 10⁶ cells) were co-cultured with fibroblasts from a patient with the same enzyme deficiency. There was no difference in enzyme activity (1.08 nmol per h per 10⁶ cells) compared with cells cultured alone (1.22 nmol per h per 10⁶ cells), whereas when co-cultured with normal lymphocytes the fibroblasts' activity increased markedly (to 4.4 nmol per h per 10⁶ cells).

Of the five lysosomal enzymes examined so far, including β -glucuronidase^{6,7}, β -glucosidase is exceptional in not being

transferred from lymphocytes. It differs from the other lysosomal enzymes in that it is tightly bound to the lysosomal membrane²⁰—the addition of the detergent taurocholate being needed to measure its activity²¹—and it is not secreted by other cells²². That β -glucosidase was not acquired by deficient fibroblasts suggests that acquisition of the other lysosomal enzymes did not occur by nonspecific phagocytosis of whole lysosomes or lysosomal debris. This was further supported by the lack of increase in the activities of any of the enzymes in the respective deficient fibroblasts when extracts of lymphocytes disrupted by freezing and thawing were added to their culture medium (I.O. and R.S., unpublished).

There was also no transfer of three non-lysosomal enzymes (Table 2): galactose 1-phosphate uridylyltransferase (EC

2.7.7.12); hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8); glucose 6-phosphate dehydrogenase (EC 1.1.1.49). This was confirmed for the first two enzymes in autoradiographic experiments using ^3H -galactose and ^3H -hypoxanthine, respectively, which showed that utilization of these precursors by deficient fibroblasts which had been co-cultured with lymphocytes was very low and equivalent to that of deficient fibroblasts cultured alone.

To confirm the specificity of this process the transfer of a fourth non-lysosomal enzyme, glucose 6-phosphate isomerase (EC 5.3.1.9), was assessed simultaneously with that of β -glucuronidase. Human fibroblasts normal with respect to the isomerase but deficient in β -glucuronidase were used as recipients, and normal mouse lymphocytes as donors. Both enzymes from mouse lymphocytes (Fig. 1, band 1) were readily distinguishable by electrophoretic procedures from those of normal human fibroblasts (band 2). Analysis of duplicate samples of the same suspension of deficient fibroblasts (band 3) after they had been co-cultured with lymphocytes (band 4), showed that only lymphocyte β -glucuronidase (Fig. 1b), and not lymphocyte isomerase (Fig. 1a), was transferred to the fibroblasts.

The mechanism of direct enzyme transfer from lymphocytes is so far unknown. The present results show that it is restricted to certain lysosomal enzymes only. Some of these are known to be present at the plasma membrane of other cell types^{23,24}, probably as a result of normal intracellular transport and membrane recycling²⁵⁻³⁰, and are secreted by them^{22,31-33}. Lymphocytes do not secrete significant amounts of any lysosomal enzyme examined so far, however, although they have significant amounts of lysosomal enzyme activities other than β -glucosidase at their plasma membrane³⁴. Direct interaction with a component of the fibroblast cell surface could potentiate the transfer of these lymphocyte enzymes and their internalization by recipient fibroblasts. The specificity of the process may be highly relevant to the effective treatment of enzyme deficiency diseases, particularly by cell transplantation procedures.

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Cell-type specific expression of a transfected immunoglobulin gene

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The introduction of cloned genes into eukaryotic cells has become a major technique in the study of gene expression. Many experiments have demonstrated transcription of cloned genes after transfection into heterologous cell systems—cells in which the genes are not normally active¹⁻⁴. More recently, several investigators have obtained expression of specialized genes after transfection into cells of the corresponding specialized type, notably the β -globin gene in erythroleukaemic cells⁵ and immunoglobulin genes in myeloma cells^{6,7}. These results allow the study of gene expression during development by comparing transcription of a gene transfected into homologous and heterologous cells. We have shown that a rearranged κ immunoglobulin gene, cloned from a mouse myeloma, is transcribed transiently at a high level when reintroduced into mouse myeloma cells⁸. We show here, in an internally controlled manner, that the same immunoglobulin gene is not detectably transcribed when transfected into mouse 3T3 or L cells.

An essential element in comparing the expression of a gene transfected into various cell types is the presence of an internal standard, a second gene that is expressed at comparable levels in all the cell lines used. To provide such a standard, we used the plasmid pSV2-neo, which contains the bacterial *neo* gene transcribed from the SV40 early promoter⁹. A rearranged κ light-chain gene, cloned from the mouse myeloma MOPC 41 (ref. 10), was inserted into this plasmid. To allow the plasmid to replicate in mouse cells, thereby increasing the levels of RNA synthesized, we also inserted a region of polyoma viral DNA containing the gene for large-T antigen and the origin of replication. The resulting plasmid pLX31 (Fig. 1) was used in all the transfection experiments described.

The plasmid pLX31 was transfected into two non-lymphoid lines of mouse cells, 3T3 and L, and one mouse lymphoid line, MPC 11, which normally secretes IgG ($\gamma 2b, \kappa$)¹¹. After 48 h, RNA was extracted from each line and assayed by the S_1 nuclease method^{12,13}. Two different 5' end-labelled probes were used for each RNA sample (Fig. 2). One probe, labelled at a site within the *neo* gene and extending upstream past the SV40 promoter, was specific for *neo* transcripts. The other probe, labelled near the 3' end of the κ gene L segment and extending upstream of the promoter, was specific for immunoglobulin transcripts.

RNA extracted from the three cell lines after transfection with pLX31 protected a long fragment of the *neo*-specific probe (Fig. 3a-c) that was not protected by RNA from mock-transfected cells (Fig. 3d,e). The size of the protected fragment corresponds to a transcript initiating from the SV40 early promoter (Fig. 2). Hence, the plasmid DNA was able to penetrate cells of all three lines in a transcribable state and direct comparable amounts of RNA synthesis from the SV40 promoter (Fig. 3a-c). The increased protection of the full-length *neo* probe by RNA from the transfected cells relative to RNA from mock-transfected cells indicates that there was also some transcription

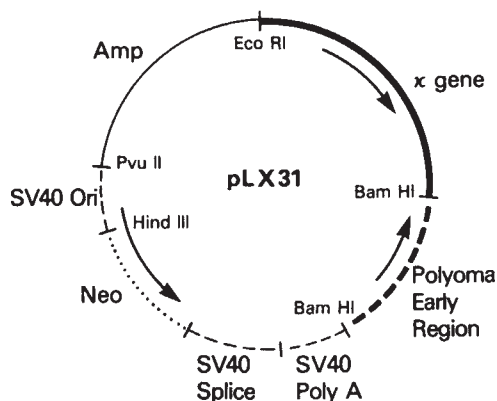


Fig. 1 Schematic diagram of the plasmid pLX31. Sources of DNA fragments are indicated as follows: pBR322 (solid line), SV40 (dashed line), *neo* gene (dotted line), polyoma (heavy dashed line), κ light-chain gene (heavy line). Structure of the pBR322-*neo*-SV40 segment is shown as described previously⁹. Relevant restriction enzyme sites are noted. Arrows indicate the approximate initiation sites and directions of transcription for the SV40 early promoter, the polyoma early promoter and the κ promoter. The figure is not drawn to scale.

Methods: All DNA fragments were purified by electroelution from agarose gels. DNA of the plasmid pSV2-*neo* was cleaved with *Eco*RI and *Bam*HI and the 4.9-kilobase (kb) large fragment purified. DNA of a phage λ clone containing the mouse MOPC 41 rearranged κ immunoglobulin gene¹⁰ was also cleaved with these enzymes, and a 7-kb *Eco*RI-*Bam*HI fragment containing the κ gene purified. The fragments were ligated, transformed into *Escherichia coli*, and a recombinant plasmid pLT31 isolated. DNA of pLT31 was cleaved with *Bam*HI and the ends were dephosphorylated. A 3.6-kb *Bam*HI fragment containing the polyoma early region, including the large-T antigen gene and origin of replication, was cleaved from another plasmid pSV5-*neo*⁹ and purified. The fragment was ligated with the prepared pLT31 DNA, transformed into *E. coli* and the recombinant plasmid pLX31 isolated. All plasmid structures were verified by restriction mapping.

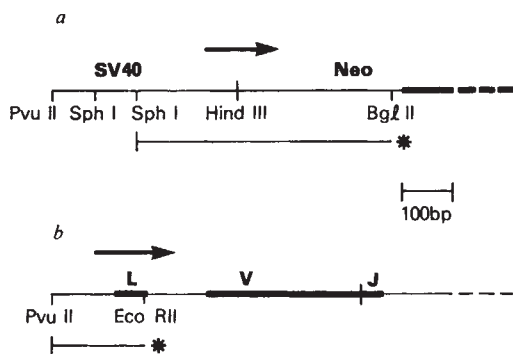


Fig. 2 Strategy for S_1 nuclease assays. The relevant DNA segments are shown schematically and restriction sites noted. The *neo*-specific (a) and κ -specific (b) probes are shown underneath the genes, with the 32 P-labelled end denoted by an asterisk. Initiation sites and direction of SV40 early (a) and κ (b) transcription are shown by arrows, and translated regions of DNA are drawn in heavy line. L, leader segment; V, variable region; J, J segment. **Methods:** The *neo* probe was prepared from pSV2-*neo* DNA and the κ probe from the plasmid pLT12, which contains the 5' part of the κ gene⁶. pSV2-*neo* and pLT12 were cut respectively with *Bgl*III and *Eco*RII, dephosphorylated, and 5'-end-labelled with [γ - 32 P]ATP and polynucleotide kinase. The kinase was heat-inactivated and the plasmids recut respectively with *Sph*I and *Pvu*II. The appropriate fragments were purified from a 4% polyacrylamide gel. Specific activities were $\sim 2 \times 10^6$ d.p.m. per pmol fragment.

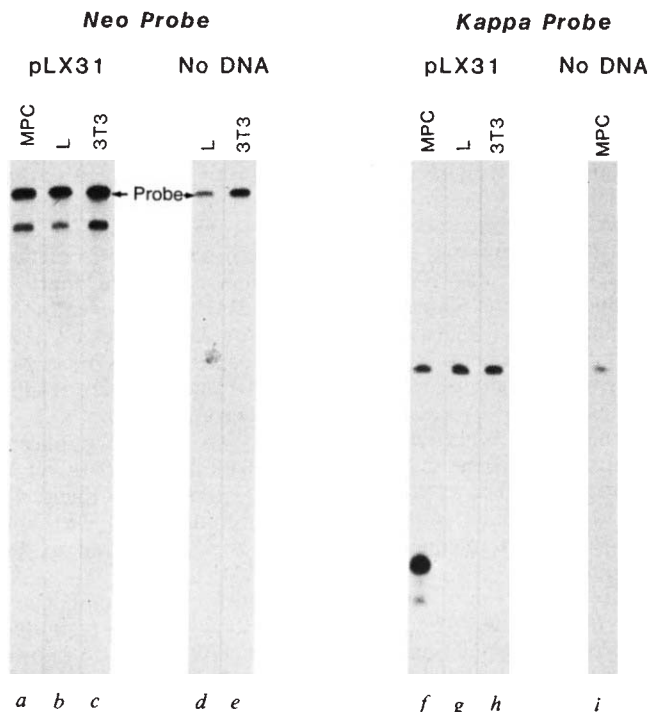


Fig. 3 S_1 nuclease assay of RNA from transfected cells. Cells of strains MPC 11, L or 3T3 were transfected with pLX31 DNA or mock-transfected (no DNA), as labelled. Total RNA was extracted from the cells after 48 h and aliquots of each RNA sample were assayed separately with both a *neo*-specific probe and a κ -specific probe. Samples were analysed together on a polyacrylamide gel. The topmost band in each lane ran at the position of undigested probe.

Methods: Cells were transfected using a modified DEAE-dextran protocol. In detail, a just-confluent 100-mm plate of cells was washed with serum-free Dulbecco's minimal essential medium, and then incubated at 37°C in 10 ml serum-free medium with 50 mM Tris pH 7.3, 250 μ g ml⁻¹ DEAE-dextran (Pharmacia, 500,000 molecular weight) and 1 μ g ml⁻¹ pLX31 DNA or no DNA (mock transfection) for 1 h (MPC 11 cells) or 3 h (L and 3T3 cells). The cells were then washed twice with serum-free media and replated in medium with serum. (The MPC 11 cells were washed by gentle centrifugation and resuspension.) RNA was extracted and partially purified by a hot acid phenol method. In detail, a plate of cells was washed with phosphate-buffered saline, suspended in 3 ml of 50 mM NaAc pH 5.2, 1% SDS, then mixed with 3 ml phenol equilibrated with 50 mM NaAc pH 5.2, and agitated for 15 min at 60°C. The solution was cooled at 0°C for 15 min, then centrifuged at 3,000 r.p.m. for 15 min. The aqueous layer was made 0.3 M in NaAc and precipitated with ethanol. The pellet was rinsed with ethanol, dried and resuspended in 150 μ l S_1 hybridization buffer¹³. The S_1 analysis was performed as described elsewhere¹³. Specifically, 20% of the RNA from each plate (representing the RNA from 3×10^6 MPC 11 or L cells or 3×10^5 3T3 cells) was hybridized with 5×10^4 d.p.m. of probe at 48°C for 12 h. Digestion was performed with 50 U S_1 nuclease (PL Biochemicals) at 37°C for 30 min. Samples were extracted with phenol/chloroform, precipitated with ethanol and run on a 4.5% polyacrylamide-urea gel. The gel was soaked for 40 min in H₂O, dried and exposed to film for 48 h with a screen.

originating upstream of the probe, probably in pBR322 sequences.

RNA extracted from MPC 11 cells after transfection with pLX31 strongly protected a fragment of the κ -specific probe (Fig. 3f) that was not protected by RNA from mock-transfected cells (Fig. 3i; RNA from the endogenous MPC 11 κ genes evidently does not hybridize with the MOPC 41 κ probe). The protected fragment is characteristic of RNA initiating at the κ promoter⁸; its size reflects a transcript starting about 25 base

Neo + Kappa

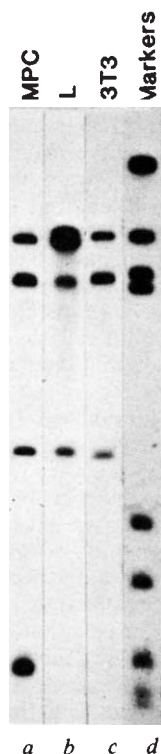


Fig. 4 S_1 nuclease assay of RNA from transfected cells. Cells of strains MPC 11, L or 3T3 were transfected with pLX31 DNA. Total RNA was extracted from the cells after 48 h and each RNA sample was assayed with an equimolar mixture of *neo*-specific and κ -specific probes. The markers are a labelled, denatured *Hinf*I digest of the plasmid pSV2-*neo*⁹ and have lengths in nucleotides of 2,263 and 1,619 (running together at the top of the gel), 517, 396, 362, 134, 109, 83 and 75 (running as a strand-separated doublet).

pairs upstream of the initiating codon for the gene. The relative amounts of *neo* and κ probe fragments protected by RNA from transfected MPC 11 cells (Fig. 3a,f) suggests that pLX31 directed at least as much transcription from the κ promoter as from the SV40 promoter, assuming equal message stability. In contrast, no transcripts from the κ promoter were detected by the S_1 assay in 3T3 or L cells transfected with pLX31 (Fig. 3g,h). Because transcription from the SV40 promoter on pLX31 serves as an internal standard for the state of the DNA template, this result implies the specific inability of a transfected immunoglobulin gene to synthesize stable transcripts in 3T3 and L cells.

To confirm our findings, we again transfected pLX31 into 3T3, L and MPC 11 cells. RNA from each line was extracted after 48 h and hybridized with an equimolar mixture of the *neo*- and κ -specific probes in an S_1 nuclease assay (Fig. 4). RNA from each line protected a comparable amount of the *neo* probe fragment diagnostic for SV40 transcription, as well as some full-length *neo* probe. Only RNA from the myeloma line protected the κ probe fragment diagnostic for transcription from the κ promoter.

Several investigators have previously transfected mouse λ or κ light-chain genes into non-lymphoid cells, notably human HeLa¹⁴ and monkey CV1¹⁵ cells. They reported no accurate transcription from the immunoglobulin promoter, as measured by transient expression assays, unless the promoter was stimulated by a nearby viral enhancer. In contrast, other groups have transfected different κ genes^{6,7}, as well as heavy-chain genes¹⁶, into mouse lymphoid cells. Using stable transformation protocols, they found that after integration in the host genome, the

transfected gene can synthesize immunoglobulin. Transient expression of an immunoglobulin gene in lymphoid cells has also been reported⁸. These results have all suggested that lymphoid and non-lymphoid cells respond differently to transfected immunoglobulin genes. However, the multiplicity of genes, species and assay methods used, as well as the absence of internal standards, have made a valid comparison of the various experiments difficult. In the experiments described here, we have controlled these elements and shown that in lymphoid cells, but not in two lines of non-lymphoid cells, a transfected κ gene produces detectable transcripts.

Our S_1 nuclease assay measures the accumulation of κ transcripts 48 h after transfection. In principle, the absence of such transcripts in non-lymphoid cells may be due either to the failure of the cells to transcribe the κ gene or to instability of the transcripts. In this regard, when transcripts of the κ gene were produced in two lines of non-lymphoid cells by attaching the gene to a viral promoter¹⁵ or by stimulating the κ promoter with a viral enhancer¹⁴, the transcripts were correctly processed and accumulated to high levels. Similarly, after transfection of a κ gene into non-lymphoid COS 7 cells, κ transcripts initiating from an aberrant startpoint were readily detected⁸. These findings strongly suggest that, once synthesized, transcripts that include the κ gene are stable in non-lymphoid cells. Hence, our present results probably indicate a failure of the non-lymphoid cells to initiate transcription of the transfected immunoglobulin gene.

The results presented here contrast with many reports that specialized genes transfected into heterologous cell systems are expressed¹⁻³. This discrepancy may partly reflect authentic differences in cellular control of different genes. Cells contain many κ variable regions, so that even low level transcription from each κ promoter in a non-lymphoid cell may be metabolically unprofitable. Hence, cells may regulate immunoglobulin promoters more tightly than promoters for other genes. Certain positive findings may also be partly explained by stimulation of the transfected gene with a viral enhancer. Thus, a transfected β -globin gene is transcribed strongly in heterologous HeLa cells in the presence of such an enhancer, but only weakly in its absence⁴. However, note that the SV40 and polyoma enhancers, in their configuration on pLX31, are evidently unable to activate transcription from the κ promoter in heterologous cells.

Two conclusions relevant to gene regulation during development can be inferred from cell-type specific expression of a transfected κ gene. First, since the DNA template is introduced in the form of an unintegrated plasmid, regulation of κ gene activity does not depend on its original chromosomal location. Second, since the κ gene was transfected into terminally differentiated cells, activation or inactivation of the gene does not depend on modifications made to it during cell development. Rather, the already differentiated cells contain all the information needed to regulate appropriately the expression of new copies of the κ gene. To what extent these conclusions apply to other genes and other cell types remains unknown.

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Efficiency of antigen presentation differs in mice differing at the *Mls* locus

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In the mouse, potent primary *in vitro* proliferation of T cells can be induced by allelic variants of cell-surface glycoproteins, Ia antigens, the genes for which are located in the I region of the major histocompatibility complex (MHC) on chromosome 17. The only other potent primary proliferative response known is induced by mixing MHC-identical lymphocytes from strains that differ at the locus termed *Mls* (ref. 1) (for mixed lymphocyte stimulating), which has been mapped to chromosome 1. While it is relatively easy to raise antibodies against Ia antigens, and thus determine both their chemical nature and their role in T-cell stimulation, the nature of the product of the *Mls* locus has remained obscure. It has been proposed that the *Mls* locus product is a minor antigen recognized in association with self-Ia antigens^{2,3}, a translocated Ia-like element⁴, or a mitogenic molecule found on the surface of antigen presenting cells (APC)⁵. Here, we demonstrate that APCs from mice carrying stimulatory *Mls* locus alleles present antigen more efficiently to cloned, antigen-specific, Ia-restricted T cells than APCs from mice carrying nonstimulating *Mls* locus alleles. We propose that the *Mls* locus does not encode a unique cell-surface antigen at all; we suggest instead that the T-cell proliferative response induced by *Mls*-locus disparate cells reflects recognition of self-Ia molecules on APCs. If our interpretation is correct, it provides further evidence both for the quantitative nature of self tolerance and for the existence of a distinct recognition site for self-Ia molecules on antigen-specific T lymphocytes.

The immunobiology of the T-cell response to cells differing at the *Mls* locus has several features that distinguish it from other T-cell proliferative responses. First, it is essentially a unidirectional response. *Mls*^{a,d} strain cells stimulate *Mls*^{b,c} strain T cells strongly, but do not respond significantly to either. *Mls*^c cells stimulate T cells of *Mls*^b strains weakly. Cells of *Mls*^b strains do not stimulate T cells of other strains significantly¹. The responder cells appear to have the cell-surface antigen phenotype *Lyt*-1⁺,2⁻ (ref. 3). Although the *Mls* locus has been mapped to chromosome 1, we have shown that monoclonal anti-I-A antibody will inhibit T-cell responses to *Mls* locus antigens³, and this has been confirmed by several laboratories⁶⁻⁸; in one recent report, both monoclonal anti-I-A and monoclonal anti-I-E largely (>80%) inhibited such responses⁸. *Mls*^a strains having both A_α:A_β (I-A) and E_α:E_β complexes (I-E) expressed on the cell surface stimulate more strongly than *Mls*^a strains lacking cell-surface expression of I-E molecules in mixed lymphocyte reactions between *Mls*-disparate strains⁹. Cloned T-cell lines can be shown to respond to *Mls*^{a,d}-bearing stimulator cells⁹⁻¹¹, and the stimulating locus can be mapped to chromosome 1 using a panel of recombinant inbred lines⁹. Although cloned, *Mls*^a-reactive T cells respond to *Mls*^a or *Mls*^d strains of several MHC genotypes⁹⁻¹¹, such clones are not stimulated by *Mls*^a stimulators of all MHC genotypes⁹. Whether this reflects a requirement for self-MHC recognition by such cloned, *Mls*-reactive T-cell lines is not known. Cloned, *Mls*-reactive T-cell lines are also very often cross-reactive on non-self MHC stimulator cells^{9,11}, particularly those bearing H-2^b. Finally, the frequency of *Mls*-reactive cells amongst resting T cells is significantly greater than the frequency of cells reactive to non-self

Ia antigens^{3,12,13}. These characteristics suggested to us that the *Mls* locus did not itself encode a cell-surface antigen, but rather that *Mls*-locus responses reflected direct recognition of self-Ia molecules by T cells. According to this hypothesis, Ia antigens on APCs of *Mls*^{a,d} strains would be more stimulatory than the identical Ia antigens on APCs from *Mls*^b strains. T cells in both *Mls*^a and *Mls*^b strains would be essentially unresponsive to their own APCs, a necessity for the maintenance of self tolerance. However, T cells from *Mls*^b strains would be responsive to the Ia antigens presented on APCs from *Mls*^a, MHC-identical mice. Thus, the T-cell response to stimulatory *Mls* alleles would be analogous to the autologous mixed lymphocyte response (AMLR)¹⁴. If this were so, one would predict that APCs of *Mls*^{a,d} strains would be more effective stimulators of cloned, antigen plus self-Ia-specific T cells than APCs from *Mls*^b strains. We have recently shown that cloned T-cell lines specific for foreign protein antigens recognized in the context of self-Ia molecules¹⁵, or specific for non-self-Ia molecules¹⁶, can be used to estimate functional levels of Ia antigen expression, and we have shown in one instance a very tight correlation of various measures of Ia antigen expression by this means¹⁷.

In the present experiments, we have used several cloned T-cell lines specific for foreign protein antigens recognized in the context of self Ia to probe antigen presentation by cells from MHC-identical, *Mls*-locus-disparate strains of mice. Our results show that for I-A^k-bearing cells, antigen presentation occurs in the order *Mls*^d ≈ *Mls*^a > *Mls*^b (Table 1; Fig. 1A). For I-A^d and I-E^d, spleen cells from *Mls*^a strains are six to seven-fold more effective APCs per cell than cells from *Mls*^b strains (Fig. 1B, C). Figure 1A and D shows that, as was previously found for I-E^k molecules¹⁵, inefficient antigen presentation can be overcome by increasing the dose of foreign protein antigen; here, about 10 times more antigen is required for similar numbers of spleen cells from *Mls*^b strains to stimulate as effectively as spleen cells from *Mls*^a strains.

Thus, data with these cloned T-cell lines suggest that *Mls* influences presentation of antigen:Ia complexes by APCs. Quantitative serological analysis of spleen cells from these same mice with monoclonal anti-Ia antibodies shows slight differences in B-cell Ia antigen expression that are far less dramatic than those observed by probing with cloned T-cell lines. By cell sorter analysis, DBA/2 spleen B cells (H-2^d, *Mls*^a) express less than double the number of I-A^d or I-E^d molecules per cell found on B10.D2 spleen B cells (H-2^d, *Mls*^b). Quantitative absorption of monoclonal anti-Ia antibodies confirms this result.

Our data suggest that functionally, Ia or antigen associated with Ia is expressed more effectively by APCs from *Mls*^a than from *Mls*^b strains of mice. This is compatible with our hypothesis

Table 1 Response of conalbumin: I-A^k specific cloned T cells to antigen with H-2^k stimulators of differing *Mls*-locus alleles

Strain	10 ⁵ Stimulator spleen cells		Cloned T-cell ³ H-thymidine incorporation	
	H-2	<i>Mls</i>	No antigen	100 µg ml ⁻¹ conalbumin
B10.BR	k	b	23	1,600
AKR/J	k	a	120	29,860
CBA/J	k	d	50	32,950

Cloned T-cell lines were prepared from AKR/J mice immunized with commercially prepared ovalbumin according to the technique of Sredni *et al.*²⁵ as modified in our laboratory²⁶. Cloned T cells plucked from agar were grown in Click's medium plus 10% fetal calf serum and 10% supernatant from concanavalin A stimulated rat spleen cells as described previously²⁶. Recloning twice by limiting dilution at high efficiency was carried out as described previously²⁶. For assay, 10⁴ washed cloned line cells were added to all wells plus 10⁵ mitomycin C inactivated spleen cells of the indicated strains. Cultures with and without 100 µg ml⁻¹ conalbumin were incubated at 37 °C in 5% CO₂ in air for 3 days, pulsed with ³H-thymidine, 1 µCi per well, for 3 h and collected. Numbers are means of triplicate counts per min; standard errors were less than 20% and have been omitted.

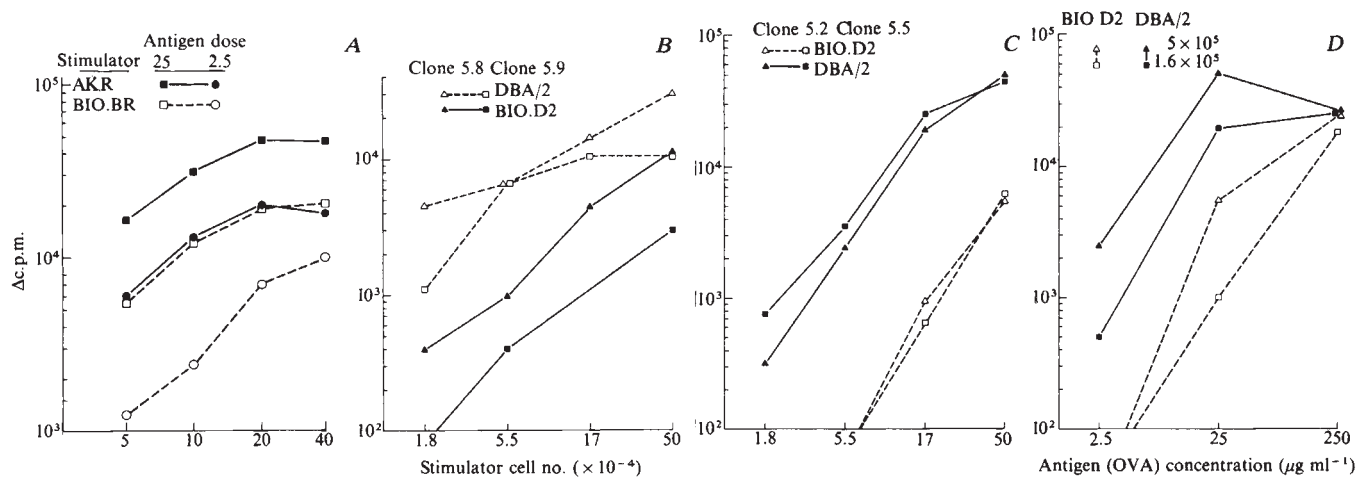


Fig. 1 Influence of *Mls* locus genotype on efficiency of antigen presentation to antigen plus self-Ia-specific cloned T-cell lines. Graded numbers of mitomycin C-treated spleen cells were added along with no antigen (control, subtracted from all responses to give Δ c.p.m.) or antigen to which the cloned T cells are specific at the dose noted on the figure. Cultures incubated for 3 days, and pulsed with $1 \mu\text{Ci } ^3\text{H}$ -thymidine for the final 3 h before collecting and counting in triplicate. Mean Δ c.p.m. presented; standard errors were less than 20% and have been omitted. **A**, Clone D10.G4.1 stimulated with graded numbers of AKR/J or B10.BR spleen cells with or without conalbumin as noted. Description of clone D10.G4.1 in Table 1 legend. Note that about five times more B10.BR spleen cells than AKR/J spleen cells are required to obtain comparable responses. Note that 10-times more conalbumin is required to obtain comparable responses at the same spleen cell dose. **B**, Clones 5.8 and 5.9, which are BALB/c OVA specific cloned T-cell lines prepared as described in ref. 26. These clones are specific for OVA in the context of I-A^d, as shown by blocking with monoclonal anti-I-A^d antibody MKD6 (P.J.C. and C.A.J., unpublished data) but not with monoclonal anti-I-E antibody 14.4.4. Graded numbers of B10.D2 or DBA/2 mitomycin C-treated spleen cells added alone or with $250 \mu\text{g ml}^{-1}$ OVA. About six times more B10.D2 than DBA/2 spleen cells required to obtain comparable responses. **C**, Clones 5.2 and 5.5, prepared as for clones 5.8 and 5.9, specific for OVA in the context of I-E^d as shown by inhibition with monoclonal anti-I-E antibody 14.4.4 but not by monoclonal anti-I-A^d antibody MKD6 (P.J.C. and C.A.J., unpublished data). OVA added at $25 \mu\text{g ml}^{-1}$. About six times more B10.D2 than DBA/2 spleen cells required to obtain comparable responses. **D**, Clone 5.2 stimulated with graded doses of OVA in the presence of two different doses of B10.D2 or DBA/2 mitomycin C-treated spleen stimulators; note that about 10 times more OVA needs to be added to obtain a given response with B10.D2 stimulators as compared with DBA/2 stimulators.

that the response to *Mls* locus antigens is in fact equivalent to an AMLR. If this were so, one might expect to find clones of antigen-specific *Mls*^b T cells that have increased responses to *Mls*^a spleen cells in the absence of added foreign antigen. Indeed, as shown in Table 2, this does occur and varies from one cloned line to the next. Some T-cell lines from *Mls*^b mice respond to *Mls*^a stimulator cells as strongly as they do to nominal antigen in the context of syngeneic *Mls*^b stimulators, and such clones may represent the normal *Mls*^b T cells that respond to *Mls*^a stimulators. Others respond weakly or not at all at this fixed

dose of stimulator cells. In a prospective study of BALB/c (*H*-2^d, *Mls*^b) OVA-specific T-cell clones, 3/11 responded like clone 10.4 (Table 2), 5/11 like clone 100.5 (Table 2) and 3/11 were intermediate. Finally, while the response of clone 10.4 to OVA on BALB/c (*H*-2^d, *Mls*^b) stimulators is blocked by a monoclonal anti-I-A^d antibody, its response to DBA/2 (*H*-2^d, *Mls*^a) stimulators in the absence of OVA is partially blocked by both monoclonal anti-I-A^d and monoclonal anti-I-E antibodies, and totally blocked by a mixture of the two. The failure of either anti-I-A^d or anti-I-E alone to block the response of

Table 2 Cloned, ovalbumin-specific *Mls*^b T-cell lines can also respond to *Mls*^a

Stimulator cells*	OVA†	Monoclonal antibody‡	Proliferative response (Δ c.p.m.) of T-cell line§			
			Expt 1		Expt 2	
			10.4	100.5	10.4	100.5
<i>H</i> -2 ^d , <i>Mls</i> ^b	—	—	(48)	(79)	(2,680)	(589)
	+	—	10,057	14,936	33,829	25,170
	+	Anti-I-A ^d	52	690	3,000	15,980
	+	Anti-I-E	10,092	13,953	24,202	30,833
	+	Both	—	—	6,630	15,486
<i>H</i> -2 ^d , <i>Mls</i> ^a	—	—	41,847	1,213	22,760	661
	—	Anti-I-A ^d	29,123	263	17,905	—
	—	Anti-I-E	6,746	44	5,895	—
	—	Both	—	—	—1,820	—
	+	—	27,058	32,701	34,254	43,008
	+	Anti-I-A ^d	24,874	5,389	—	23,719
	+	Anti-I-E	23,011	30,986	—	38,275
	+	Both	—	—	—	20,353

* Stimulator cells: 2×10^5 mitomycin C-treated spleen cells. Expt 1, *H*-2^d, *Mls*^b = BALB/cByJ; Expt 2, *H*-2^d, *Mls*^b = B10.D2. Expts 1 and 2, *H*-2^d, *Mls*^a = DBA/2J.

† Ovalbumin added at $100 \mu\text{g ml}^{-1}$ final concentration.

‡ Monoclonal antibodies: Anti-I-A^d = MKD6, anti-I-E = 14.4.4. Monoclonal antibodies added at a final dilution of 1:80 of a purified hybridoma ascites fluid. Both: MKD6 and 14.4.4 added at 1:160 each.

§ Cloned, BALB/cByJ OVA-specific T-cell lines prepared as noted in Fig. 1. Data represent the mean ^3H -thymidine incorporated during a 4-h pulse on day 3 of triplicate cultures by 4×10^4 cloned T cells. Background without antigen on *H*-2^d, *Mls*^b stimulator cells given in parentheses and subtracted from all other values.

clone 10.4 to OVA presented by H-2^d, *Mls*^a stimulators most likely reflects the dominance of I-A^d in the response of 10.4 to OVA, and of I-E^d in the response to *Mls*^a. Thus, the response of clone 10.4 to *Mls*^a resembles the response of normal BALB/c T cells to *Mls*^a in this regard⁸.

Note that our findings thus far apply only to certain strains of mice that differ at the *Mls* locus. Because the strains we have used in these studies are those for which the phenomenology of the *Mls* locus is most firmly established, we have described our findings, and will discuss them, as though the effects were indeed due to the *Mls* locus itself. This needs to be determined in subsequent studies.

We believe that quantitative variation in the ability of APCs to present antigen:Ia complexes and, by inference, self-Ia antigens, accounts for the primary T-cell proliferative responses seen between MHC-identical, *Mls*-disparate strains. First, it would account for the unidirectional nature of the response and for the direction in which that response occurs. Second, it would account for the greater responses to *Mls*^a seen when the stimulators express both I-A and I-E molecules, since *Mls*-locus-reactive T cells apparently respond largely to non-polymorphic portions of Ia molecules⁸⁻¹¹, and since I-E-positive cells would express more Ia molecules than would I-E-negative cells. This is strongly supported by the monoclonal antibody blocking studies in Table 2. Third, it would account for the very high precursor frequency of *Mls*^{a,d}-reactive T cells, since essentially all *Lyt*-1⁺2⁻ T cells are thought to have receptors for self-Ia molecules, and a subset of these can react to these molecules in the apparent absence of foreign antigen (Table 2).

Several interesting implications follow from our interpretation of these and previous data on responses to *Mls* locus antigens. First, they suggest that self tolerance to Ia glycoprotein antigens is quantitative rather than qualitative. A similar finding has already been reported by three groups working with cytolytic T cells^{18,19} or antibody responses²⁰ to class I MHC antigens. Thus, T cells would have a receptor site for self Ia that is selected during ontogeny to bind Ia expressed on autologous APC with an affinity just below that required to activate the cell to proliferate^{21,22}. Second, if T-cell responses to *Mls* represent self-Ia antigen recognition, and if such T cells do not discriminate self from non-self Ia (Table 2)⁹⁻¹¹, this may suggest that the receptor site for self Ia recognizes a non-polymorphic portion of the Ia molecule. If this is so, then two interesting corollaries follow. The first is that the T cell must have an independent recognition site for antigen, in which case MHC restriction in antigen recognition would involve the association of antigen with a polymorphic portion of the Ia molecule, as we had argued previously for responses of T cells to haptens on chemically distinct carrier molecules²¹. This interpretation receives strong support from the studies of Heber-Katz *et al.*²³ on T-cell responses to pigeon cytochrome c. Finally, if this construction is correct, then the observation that the Ia genotype of the thymus dictates self-Ia recognition by T cells implies that not only self Ia but also some surrogate for foreign antigen, such as self idiotopes or self cell-surface antigens on thymic epithelial cells may have a role in generating the MHC restriction that is characteristic of mature *Ly* 1 T cells²⁴.

The mechanism(s) by which antigen:Ia complexes are more efficiently presented by *Mls*-stimulating APCs remains to be determined, as does the mechanism by which non-reactivity to self is so precisely regulated. We believe that analysis of the *Mls* locus will permit valuable insights into the mechanism of self tolerance and its breakdown, as well as the generation of the T-cell repertoire, most especially of the recognition site for self-Ia molecules.

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Organization and bidirectional transcription of H2A, H2B and H4 histone genes in rice embryos

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Our current understanding of the evolution of the histone gene family suffers from a lack of information on plant histone genes¹. With a view to gathering some much needed information on these genes, we studied a rice genomic clone in pBR322 carrying H2A, H2B and H4 histone genes on a DNA fragment² of 6.64 kilobases (kb). A restriction map of the insert was constructed and the organization of the three genes on this insert was determined. H2A and H2B histone genes were located at one end of the insert and H4 gene at the other with a 3.1 kb spacer in between. This cluster of three histone genes was found to be transcribed in a bidirectional fashion with H2A and H2B genes being encoded by one strand and the H4 gene by the other. These results indicate that plant histone gene organization differs from that of the sea urchin, but shows many similarities to the systems in other animals.

Seed germination is a process where a dormant tissue is suddenly activated into a metabolically vigorous one where, as in the case of all developing tissues, there is a tremendous demand for histones. We already know of two means by which the rice embryos ensure an adequate supply of histones for its developmental needs—a free pool of histones in the nucleosol of dry embryos³, and a pool of conserved messengers for all the histones, present in the cytosol⁴. (This is similar to the system in sea urchins and amphibians, where pools of histones^{5,6} or their messages⁷⁻¹¹ are present.) So far, we have had no information on the regulation of histone gene expression in the course of germination. For this an understanding of the histone gene organization is a primary requirement. As a first step, we cloned *Bam*HI fragments of 48-h germinated rice (*Oryza sativa* L. IR20) embryo DNA in pBR322 and identified one clone carrying H2A, H2B and H4 histone genes².

The 6.64-kb-long insert DNA from the clone pIR22 was labelled at the 5'-ends with T4 phage polynucleotide kinase and [γ -³²P]ATP¹², cleaved with single-cut enzyme *Eco*RI and a restriction map constructed according to Smith and Birnstiel¹³.

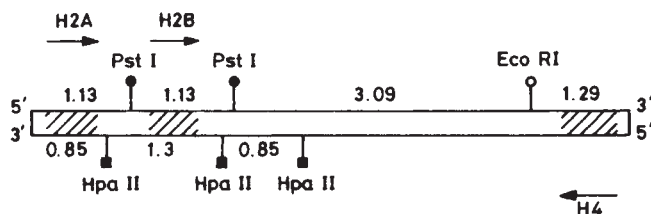


Fig. 1 Restriction sites for *Pst*I, *Eco*RI and *Hpa*II enzymes on the insert. A restriction map of the insert was constructed using eight enzymes by the Smith and Birnstiel method¹³. These three enzymes were selected so as to obtain overlapping fragments of the insert. The sizes are indicated in kilobase pairs. Hatched areas represent the location of the histone genes (see Fig. 2).

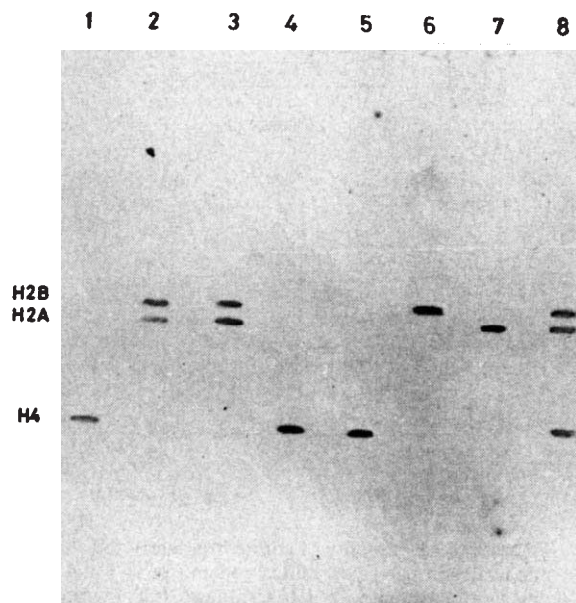


Fig. 2 Fluorogram of the translation products of RNAs that hybridized to *Pst*I, *Eco*RI and *Hpa*II restriction fragments of the insert. The various fragments obtained after digestion with these three enzymes were separated on a 1.5% agarose gel, blotted on to a nitrocellulose filter by the Southern technique¹⁴ and the portions corresponding to the bands cut out and hybridized against 18-h germinated rice embryo RNA². The hybridized RNA was eluted and translated in an *in vitro* system derived from dry rice embryos⁴ using ³⁵S-methionine². The products were precipitated with trichloroacetic acid and separated by electrophoresis on an acid-urea 15% polyacrylamide gel¹⁵. Translation products of RNAs which hybridized to DNA fragments obtained after restriction with: lane 1, *Pst*I (4.38 kb); lane 2, *Pst*I (1.13 kb, 1.13 kb); lane 3, *Eco*RI (5.35 kb); lane 4, *Eco*RI (1.29 kb); lane 5, *Hpa*II (3.64 kb); lane 6, *Hpa*II (1.30 kb); lane 7, *Hpa*II (0.85 kb, 0.85 kb); lane 8, unrestricted insert.

We selected three restriction endonucleases, *Pst*I, *Eco*RI and *Hpa*II, which would give sufficient overlapping sequences to locate the three genes on the insert (Fig. 1). The fragments generated by these enzymes were separated on agarose gel and transferred to nitrocellulose filters by the Southern blot method¹⁴. RNAs hybridizing to the various fragments were captured in stringent conditions of hybridization², eluted and translated in a homologous cell-free system using ³⁵S-methionine². The products were analysed on a Panyim and Chalkley gel¹⁵ and fluorography was done according to Laskey¹⁶ (Fig. 2). ¹⁴C-labelled *Chlorella* protein hydrolysate, when used as the radioactive label instead of ³⁵S-methionine, gave identical products showing that all the proteins encoded by the insert contain methionine. All the radioactivity in the translated prod-

Fig. 3 Strand separation of the insert. This was done according to Gross *et al.*²³. Lane 1, denatured DNA; lane 2, native DNA.

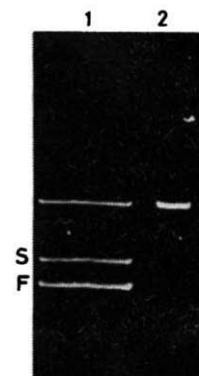
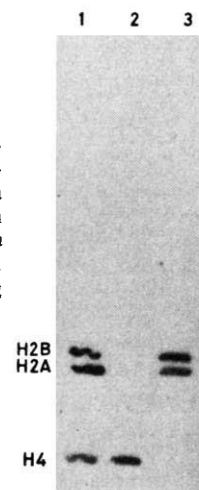


Fig. 4 Fluorogram of the translation products of RNAs hybridizing to the two separated strands. The DNA was transferred to a nitrocellulose filter, hybridized against 18-h germinated rice embryo RNA, translated *in vitro* and the products analysed as for Fig. 2. Translation products of RNAs hybridizing to: lane 1, insert; lane 2, fast-moving strand; lane 3, slow-moving strand.



ucts was recovered in the three bands corresponding to H2A, H2B and H4, indicating that this insert does not code for any other protein. From Figs 1 and 2 it is clear that the H2A histone gene is found in the 0.85 kb *Hpa*II fragment, H2B in the 1.13 kb *Pst*I and 1.3 kb *Hpa*II fragments at one end, and H4 in the 1.29 kb *Eco*RI fragment at the other end. This study, however, does not indicate the exact sizes of these genes. Sequencing data (not shown here) indicate that H2A gene occurs within 300 nucleotides from the 5'-end of one strand.

Histone genes are generally G + C-rich with A + T-rich spacers between them¹⁷. From the restriction map using G + C-specific enzymes, we know that the terminal portions of the insert are G + C-rich (data not shown). The location of the genes at the two ends indicates that histone genes, as in the case of many other systems, are also G + C-rich.

Sea urchins differ from many other organisms in their histone gene organization. One difference is that sea urchins have unidirectional transcription whereas in *Xenopus*¹⁸, newt¹⁹, chicken^{20,21} and yeast²² the histone genes are located on both strands, necessitating bidirectional transcription¹. To examine the way in which plant histone genes are organized, we separated the strands of the insert after alkali treatment on an agarose gel²³ (Fig. 3), transferred them to a nitrocellulose filter, captured mRNAs by hybridization, translated the RNA *in vitro* and analysed the products (Fig. 4) as before. The transcription of the three rice histone genes was found to be bidirectional—H2A and H2B genes being encoded by one strand and H4 gene by the other.

From the above data, it is clear that two histone genes H2A and H2B are located at the 5'-end of one strand and H4 gene at the 5'-end of the other strand, leaving a spacer of about 3.1 kb in between (Fig. 1). Work is now under way to determine the organization of the other histone genes and the extent of repetition of the histone gene family in rice genome.

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Transcriptional regulation of growth hormone gene expression by growth hormone-releasing factor

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Production and release of hormones by the pituitary is known to be under complex hormonal control. Prolactin, for example, is both positively and negatively regulated by steroid and thyroid hormones as well as by peptide hormones^{1–5}. Some hypothalamic releasing factors have been shown to regulate both hormone biosynthesis and hormone release^{6–11}. In the case of growth hormone (GH), glucocorticoids and thyroid hormone stimulate its production^{12–14}, at least in part by stimulating transcription of the GH gene¹², and somatostatin inhibits^{6,15,16} and growth hormone-releasing factor (GRF) stimulates^{17–19} its release. So far, however, polypeptide hormone regulation of GH biosynthesis has not been demonstrated. Recently a peptide with GH-releasing activity has been characterized from human pancreatic islet tumours^{14,20,21}. We report here that pure human pancreatic GRF (hpGRF) stimulates transcription of the GH gene, as well as stimulating GH release.

Primary cultures of rat anterior pituitary cells were grown in medium containing 5 nM dexamethasone and 30 pM triiodothyronine⁸. After 3–4 days in culture, the cells were incubated for 1 h in serum-free medium with or without 10 nM hpGRF. After the GRF treatment, nuclei from the cells were isolated and nascent RNA chains were allowed to elongate for 50 min in the presence of ³²P-labelled UTP and CTP, as pre-

viously described⁶. RNA was purified and transcription rates for specific genes determined by hybridization to cDNA plasmids. Hybridization was carried out in DNA excess, which was verified by adding increasing amounts of labelled RNA to a series of hybridizations and observing proportionately increased hybridization values (data not shown).

In the conditions used for labelling, RNA polymerase molecules will not re-initiate transcription, so the amount of label incorporated into GH RNA is a measure of the number

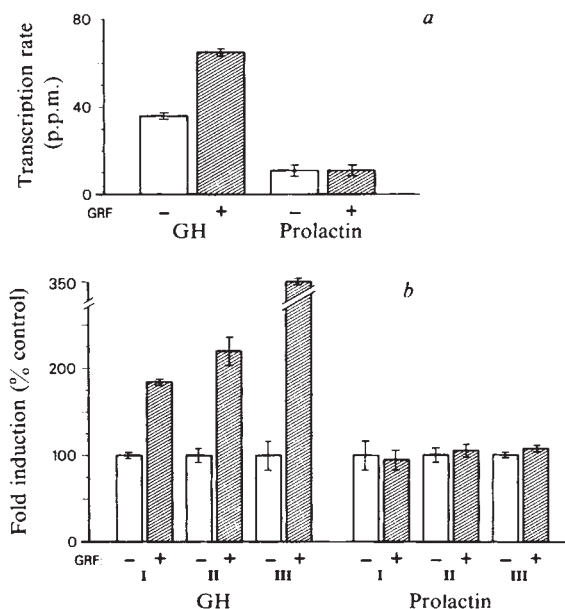


Fig. 1 Transcriptional response of cultured pituitary cells to GRF. *a*, Transcription rates in parts per million (p.p.m.) of GH and prolactin genes, with (+) and without (-) GRF treatment. *b*, Transcription rates in the presence (+) of GRF expressed as per cent of control (-) for three separate experiments.

Methods: Primary cultures of anterior pituitary cells were prepared from male Sprague-Dawley rats, as described previously^{7,14} and plated at a density of 10×10^6 cells per 10-cm dish in medium containing 5 nM dexamethasone and 30 pM triiodothyronine. After 3 days in culture the cells were washed by multiple medium change with B-PJ medium containing 1% bovine serum albumin and 2.5×10^{-4} M ascorbic acid¹⁴ and returned to the incubator. After 1-h incubation they were treated with 10 nM hpGRF in the same medium. Control cells were mock-treated by addition of GRF dilution buffer (1 mM acetic acid). After 1 h of treatment, cells were washed with cold phosphate-buffered saline, lysed in 100 mM KCl, 10 mM Tris pH 7.9, 5 mM MgCl₂, 0.1 mM EDTA, 0.1% NP-40, 5 mM dithiothreitol, 0.25 M sucrose, homogenized in a Dounce tissue homogenizer and centrifuged at 1,500g in a J6B clinical centrifuge for 3 min to pellet nuclei. The nuclei were incubated in ³²P-labelled UTP and CTP to elongate nascent RNA chains¹². Radioactive RNA was prepared from the nuclei and hybridized to excess plasmid DNA fixed on nitrocellulose filters¹². The filters were washed with RNase treatment¹², dried and counted in toluene scintillation fluid. GH gene transcription was measured by quantifying RNase-resistant hybridization to a full-length GH cDNA clone. A prolactin intervening sequence (IVS) subclone was used to quantify prolactin transcription. The IVS clone gives the same transcription rate determinations as a prolactin cDNA clone, when corrected for probe length, and was used because it is longer than the cDNA clone. Background hybridization was determined by hybridization to pBR322, and the background hybridization values were subtracted from the other hybridization values to give specific hybridization. GH- and prolactin-specific hybridization is presented as p.p.m. of total input RNA. In reporting the data no corrections are made for the size of the hybridization probe or the efficiency of hybridization. Input for this experiment was about 20×10^6 c.p.m. per hybridization. The GH- and prolactin-specific transcription was inhibited when α -amanitin ($5 \mu\text{g ml}^{-1}$) was included during the labelling period. Hybridizations were done in triplicate and data are presented as the mean $\pm$ s.e.

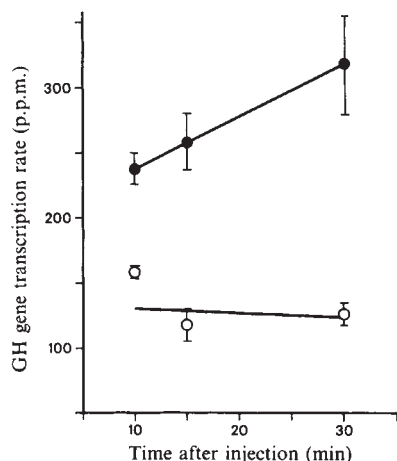


Fig. 2 *In vivo* response of GH gene transcription rate to GRF injection. GH gene transcription rates in p.p.m. were determined in pituitaries isolated from anaesthetized rats 10, 15 or 30 min after injection of hpGRF (●) or saline (○). Determinations were done on either individual pituitaries or pairs of pituitaries from similarly treated rats. The 15- and 30-min points represent the mean $\pm$ s.e. of hybridization values for four individual rats. Two individual rats were used for each 10-min determination.

Methods: Male Sprague-Dawley rats weighing 230–240 g were anaesthetized with Nembutal (40 mg per kg intraperitoneally) to suppress GH surges²³. Fifteen minutes after anaesthesia they were injected with hpGRF (100 μ g per kg intravenously) or saline. At 10, 15 or 30 min after GRF injection, the rats were decapitated, bled and the pituitaries removed and immediately frozen in liquid nitrogen. Later the frozen pituitaries were homogenized in a Dounce tissue homogenizer, in the lysis buffer described in Fig. 1 legend. Nuclei were labelled and assayed for transcription rates as described in Fig. 1 legend. Serum GH levels were determined by radioimmunoassay. When two pituitaries were pooled for transcription assay, hybridization was done in triplicate, and when individual pituitaries were assayed, duplicate hybridizations were carried out.

of RNA polymerase molecules on the gene at the time the nuclei are collected. This direct measurement of transcription rate is the only experimental approach currently available to establish unequivocally that an agent is regulating gene expression at the level of transcription. Determinations of RNA levels may reflect rates of RNA processing, transport and degradation, as well as transcription rates, and therefore are not reliable indicators of transcriptional regulation.

GRF treatment increased the transcription rate of the GH gene to almost twice that observed in untreated cells (Fig. 1*a*). The rate of prolactin gene transcription does not change in response to GRF treatment. As further control, the transcription rate was measured for *CHOB*, a gene which is expressed constitutively in all mammalian tissues and cell lines so far examined²². The *CHOB* transcription rate was about 10 p.p.m., and did not change in response to GRF treatment (data not shown). This demonstrates that GRF does not regulate this constitutively expressed gene, and therefore suggests that GRF is not having a general effect on transcription rates, but rather is specifically affecting the GH gene.

Figure 1*b* shows the results of three experiments of similar design to the experiment in Fig. 1*a*. The data have been normalized to show the transcription rate for each GRF treatment as a per cent of the control value for that experiment. In each experiment there is a statistically significant induction of GH transcription ($P < 0.01$) and the magnitude of the induction varies from 1.8-fold to 3.6-fold. For all the experiments in Fig. 1, GRF treatment lasted 1 h. When cells were treated with GRF for 30 min, a 1.8-fold transcriptional induction was observed (data not shown), demonstrating that the transcriptional effect is rapid.

We have further demonstrated that GRF regulates transcription of the GH gene *in vivo* as well as *in vitro*. Rats were anaesthetized with Nembutal, which represses endogenous GH surges²³. Fifteen minutes after anaesthesia, the rats were injected intravenously with hpGRF. The rats were decapitated 10, 15 or 30 min after the GRF injection, blood samples were taken and pituitaries were removed for transcription rate analysis. Plasma GH levels were determined by radioimmunoassay. Figure 2 shows that, *in vivo*, GRF increases GH gene transcription by ~ 1.8 -fold at 10 min and by 2.5-fold at 30 min after injection. In contrast, GH levels in the blood reach a maximum level, 11-fold over control, within 15 min of GRF injection.

Our experiments show that GRF regulates GH expression at the level of transcription, and that this transcriptional regulation occurs both *in vivo* and *in vitro*. Our data suggest that the transcriptional response is a rapid receptor-mediated event, and are consistent with the notion that both release and transcriptional induction are independent events, occurring simultaneously within the cell. The magnitude of the transcriptional induction observed is approximately twofold, which is consistent with the 1.75-fold increase in GH content of cells and medium observed after long-term exposure of cells to GRF⁶. It seems, therefore, that transcriptional induction is responsible for the observed effect of GRF on GH synthesis. The physiological significance of this transcriptional regulation is that it allows 'fine tuning' of GH gene activity, over and above the transcriptional regulation by glucocorticoids and thyroid hormone.

With these experiments, we have used direct measurement of transcription rates to provide the first evidence of transcriptional regulation by a hypothalamic regulatory peptide in normal pituitary cells. Unlike the various pituitary tumour cell lines which do not store GH, and therefore cannot demonstrate a true release response, the primary cultures of normal pituitary cells do store GH in secretory granules and release these granules in response to GRF. Since normal pituitary cells demonstrate both the release and transcriptional responses to GRF which we have shown to occur *in vivo*, they should be a suitable model system for the further study of the mechanisms of both these responses.

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Expression-cloning and sequence of a cDNA encoding human growth hormone-releasing factor

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Growth hormone-releasing factor (GRF) is believed to mediate both neural and hormonal control of the release and perhaps synthesis of growth hormone (GH) from the anterior pituitary. Its presence in only minute quantities in the hypothalamus hampered for many years efforts to determine its structure. Recently, two groups independently characterized a peptide from two human pancreatic tumours that possessed GH-releasing activity^{1,2}. Antibodies to this peptide have revealed immunoreactive material in the hypothalamus of several primate species³. Furthermore, an apparently identical peptide has now been isolated directly from human hypothalamus⁴. Using both oligonucleotide⁵⁻⁷ and antibody⁸⁻¹⁰ screening of a cDNA expression library, we have isolated a recombinant clone encoding human pancreatic GRF (hpGRF) within a larger precursor protein. The nucleotide sequence predicts that processing of GRF from the precursor should generate two additional peptides of unknown function. Restriction analysis of genomic DNA indicates that there is probably a single human GRF gene and suggests that the pancreatic tumour and hypothalamic proteins are encoded by an identical mRNA.

To determine whether antiserum against GRF peptide would react with a GRF precursor protein, poly(A)⁺ RNA from a human pancreatic tumour ectopically producing GRF was translated *in vitro* in a rabbit reticulocyte lysate. The labelled translation products were immunoprecipitated with antibody prepared against synthetic GRF(1-40)-OH and the immunoprecipitated products visualized by polyacrylamide gel electrophoresis and autoradiography (Fig. 1). A single prominent immunoprecipitation product of molecular weight (MW) ~13,000 was identified. Precipitation of this product was inhibited by the addition of excess unlabelled synthetic GRF(1-40)-OH, and pre-immune serum did not precipitate this product (Fig. 1). Because the 13,000-MW product is two to three times larger than expected for the 44-amino acid GRF peptide, these results indicate that GRF is synthesized as a larger precursor protein and that the antiserum recognizes this precursor.

Double-stranded cDNA was synthesized using size-fractionated poly(A)⁺ RNA from the GRF-producing tumour as template. cDNA synthesis and ligation into bacterial expression vector pUC8 (ref. 11) were as described previously⁸. Starting with 2 µg of size-selected poly(A)⁺ RNA, 100 ng of double-stranded, linked cDNA was obtained. Transformation of *Escherichia coli* strain DH-1 with this cDNA yielded approximately 50,000 independent colonies, 95% of which contained inserts. Approximately 8,000 recombinant colonies were replica-plated in duplicate onto nitrocellulose filters. One set of filters was processed by the method of Grunstein and Hogness¹² for eventual screening with an oligonucleotide pool. The oligonucleotides chosen correspond to amino acids 24-27 of GRF and are listed in Fig. 2 legend. Based on both observed codon usage¹³ and the observation that a G-T base pair does not significantly alter the stability of a short hybrid¹⁴, we chose to use a G in the synthetic oligonucleotide at positions corresponding to both U and C in the mRNA. This reduced the complexity of the pool fourfold. The second set of filters was processed by the method of Helfman *et al.*⁸, for immunological

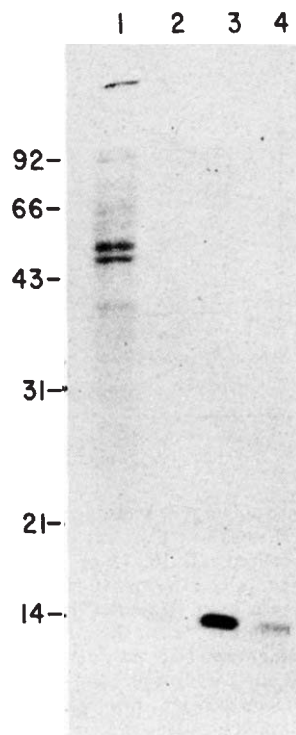


Fig. 1 Immunoprecipitation of translation products directed by GRF tumour mRNA. Total RNA was isolated from a portion of a human pancreatic tumour possessing GH-releasing activity¹⁹ using standard techniques. Poly(A)⁺ RNA was isolated by a single cycle of adsorption to and elution from oligo(dT)-cellulose (Collaborative Research). Individual translation reactions contained 9 µl mRNA-dependent reticulocyte lysate²⁰, 15 µCi ³⁵S-methionine (NEN) and 50 ng poly(A)⁺ RNA. After incubation at 30 °C for 90 min, reactions were diluted to 100 µl with stop buffer (phosphate-buffered saline, 2% Triton X-100, 10 mM L-methionine), 10 µg unlabelled GRF(1-40)-OH was added to appropriate samples, and 1 µl of either pre-immune serum or rabbit anti-GRF serum G57 was added. Antiserum G57 was raised against GRF(1-40)-OH glutaraldehyde cross-linked to human α-globulins and was pre-absorbed with human α-globulins (unpublished results). After incubation at 4 °C overnight, 150 µg pre-washed formalin-fixed *Staphylococcus aureus* bacteria were added, samples were incubated for 1 h at room temperature, and the precipitate collected, washed and resuspended in gel sample buffer. The immunoprecipitates were electrophoresed on 12% polyacrylamide-SDS gels²¹ and the gels processed for fluorography with Enhance (NEN). Lane 1, total translation products (one-tenth of reaction); lane 2, precipitation with pre-immune serum; lane 3, precipitation with anti-GRF serum; lane 4, precipitation with anti-GRF serum in the presence of excess unlabelled GRF. MW markers (×10³) are from top to bottom: phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme.

screening. The initial screening yielded 10 oligonucleotide-positive colonies and 5 immunologically positive colonies. These, along with several negative colonies, were picked and replica-plated in a registered fashion for secondary screening. Figure 2 shows the results obtained with one duplicate set of filters. A single colony on this filter was positive in both the oligonucleotide hybridization and immunological expression assays. This recombinant clone, termed phGRF(1-54), was chosen for further analysis.

Figure 3a shows the complete nucleotide sequence of the 509-base pair (bp) insert from phGRF(1-54). The strategy used to determine the nucleotide sequence is presented in Fig. 3b, together with a schematic representation of the structure of clone phGRF(1-54). An open reading frame extends from the

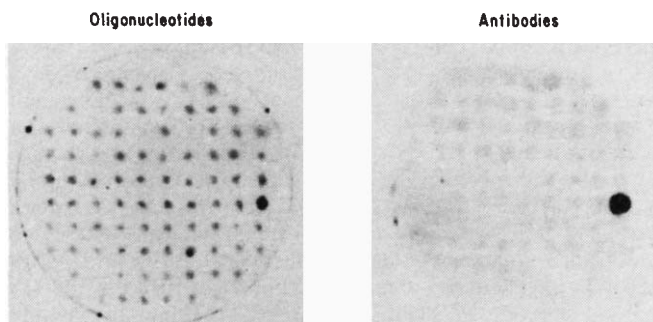


Fig. 2 Identification of a human GRF cDNA clone. Poly(A)⁺ RNA from the GRF-containing tumour was size-fractionated on a 5–20% sucrose gradient and fractions smaller than 18S were pooled and used for cDNA synthesis. First-strand cDNA synthesis was performed using oligo(dT) and avian myelomatosis virus (AMV) reverse transcriptase (Bethesda Research Laboratory), and second-strand synthesis using both the large fragment of DNA polymerase I (New England Biolabs) and AMV reverse transcriptase as described elsewhere²². *SalI* and *EcoRI* synthetic linkers (New England Biolabs) were ligated sequentially to the 3' and 5' ends of the double-stranded cDNA respectively, as described previously²². After digestion with the restriction enzymes *EcoRI* and *SalI*, cDNAs larger than 300 bp were selected by chromatography on Sepharose 4B. The plasmid pUC8 (ref. 11) was digested with *EcoRI* and *SalI* and the 16-bp fragment separated from the vector by electrophoresis in 1% agarose. Individual ligation reactions containing 10 ng cDNA and 100 ng pUC8 vector were performed overnight at 10 °C, and aliquots containing 20 ng DNA were used to transform 100- μ l aliquots of *E. coli* DH-1 that had been made competent with CaCl_2 ²³. Bacteria were plated on ampicillin plates at a density of 500 colonies per plate and were replica-plated in duplicate to 82-mm nitrocellulose filters (Schleicher and Schuell) for subsequent screening. Four independent dodecamers (5' CATGATGTCTTG 3', 5' CATTATGTCTTG 3', 5' CATGATGTCCTG 3', 5' CATTATGTCCTG 3') were synthesized by conventional triester methodology²⁴. Each was labelled using [γ -³²P]ATP and T4 polynucleotide kinase (Bethesda Research Laboratory) and ethanol-precipitated twice in the presence of carrier salmon sperm DNA. For screening of colonies, equal amounts of each oligonucleotide were pooled. Conditions for bacterial lysis on nitrocellulose filters for immunological screening were as described previously⁸. The IgG fraction was isolated from 1 ml anti-GRF serum G57 by Protein A-Sepharose affinity chromatography²⁵, resuspended in 1 ml saline (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) and incubated with the filters being screened at a dilution of 1:250 for 1 h at room temperature. After washing, the filters were incubated with 10 μCi ¹²⁵I-Protein A (9.7 μCi μg^{-1} , NEN) for 1 h at room temperature. The filters were washed for several hours in saline +0.1% Triton X-100 and autoradiographed. In each round of screening, a control filter on which GRF standards (1 ng–1 μg) had been spotted was included. Areas surrounding several potential positive clones from the initial high-density screen described in the text were picked onto grids (88 colonies per 82-mm plate), replica-plated in duplicate on nitrocellulose filters and screened in parallel with the pool of oligonucleotides (left) or with antibodies against GRF (right).

a

5'-ATG ACC ATG ATT ACG AAT TCC
EcoRI

TTC TTC TTT GTG ATC CTC ACC CTC AGC AAC AGC TCC CAC TGC TCC 45
phe phe phe val ile leu thr leu ser asn ser ser his cys ser

CCA CCT CCC CCT TTG ACC CTC AGG ATG CGG CGG TAT GCA GAT GCC 90
pro pro pro leu thr leu arg met arg arg tyr ala asp ala

ATC TTC ACC AAC AGC TAC CGG AAG GTG CTG GGC CAG CTG TCC GCC 135
ile phe thr asn ser tyr arg lys val leu gly gln leu ser ala

CGC AAG CTG CTC CAG GAC ATC ATG AGC AGG CAG CAG GGA GAG AGC 180
arg lys leu leu gln asp ile met ser arg gln gln gly glu ser

AAC CAA GAG CGA GGA GCA AGG GCA CGG CTT GGT CGT CAG GTA GAC 225
asn gln glu arg gly ala arg ala arg gly arg gln val asp

AGC ATG TGG GCA GAA CAA AAG CAA ATG GAA TTG GAG AGC ATC CTG 270
ser met trp ala glu gln lys gln met glu leu glu ser ile leu

GTG GCC CTG CTG CAG AAG CAC AGC AGG AAC TCC CAG GGA TGA AGA 315
val ala leu leu gln lys his ser arg asn ser gln gly

TTC CTC CTG TGA CCC GGG CTA CCT GTA GCC AAA ATG CAA CTG GAT 360
CCA GTT AAT CCT CTC ATT TCT GAC CCA CTT TTT CCT TTG AAA ATA 405
CAA TAA AAT TCC CCC A₍₈₈₎ G GTC GAC C -3'
SalI

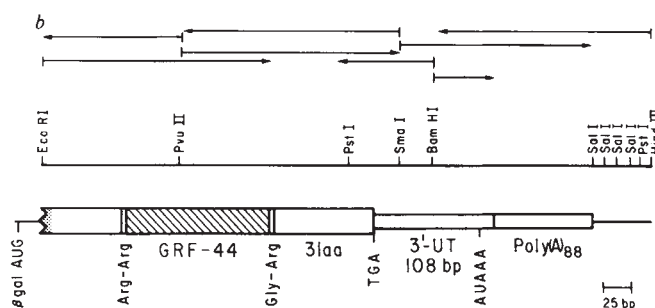


Fig. 3 a, Nucleotide sequence of the insert from clone phGRF-54. Restriction fragments were end-labelled using [γ -³²P]ATP and polynucleotide kinase. Sequence determination was by the method of Maxam and Gilbert²⁶. *EcoRI* and *SalI* designate the synthetic linkers used for cloning. Nucleotides 1–309 contain an open reading frame that is translated into the amino acid sequence shown. The amino acid sequence of GRF(1–44) is underlined. Amino acids presumed to be involved in processing of GRF from the precursor are boxed. Only the sense-strand sequence is shown, although both strands have been completely sequenced. b, Schematic representation of the insert from clone phGRF(1–54). The restriction map in the centre shows the location of sites for the 6-bp cutters used in the sequence determination. The four *SalI* sites at the 3' end of the insert presumably are a result of incomplete digestion following the ligation of the *SalI* linker during the cloning process. The lines above the restriction map depict the strategy used to determine the nucleotide sequence presented in a. The structure of the cDNA insert is presented schematically in the lower part of the figure. The shaded area represents a portion of the putative GRF signal sequence (see text). The cross-hatched area is the coding region for GRF(1–44). The positions of identified initiation, termination and processing signals are shown. 3'-UT is the 3'-non-translated region. aa, Amino acids.

β -galactosidase initiation codon within the vector to nucleotide 309 of the insert, and encodes the entire sequence of GRF(1–44). The open reading frame is followed by 108 nucleotides of 3'-non-translated RNA and an 88-residue poly(A) tract. The nucleotide sequence of our cDNA indicates that, as expected, GRF is contained within a larger precursor protein. The MW estimate of 13,000 for the GRF precursor suggests it should be approximately 110 amino acids long. The open reading frame within phGRF(1–54) encodes 103 amino acids. We therefore estimate that this cDNA is missing about 20 nucleotides of translated sequences in addition to the 5'-non-translated sequences. This is consistent with the observation that the amino-terminal six amino acids are all hydrophobic, suggesting

that the sequence extends into the signal peptide of the GRF precursor, which is expected to be a secreted protein.

In general, processed peptides are flanked by paired basic residues which serve as cleavage sites for serine protease processing enzymes¹⁵. Peptides that are C-terminally amidated are immediately followed by a glycine that probably serves as the amide donor¹⁶. GRF appears to have this same general structure with one exception; there is a single basic residue (arginine) at the C-terminal end of the peptide. Other processing cleavages at single basic residues occur in the arginine vasopressin-neurophysin precursor¹⁷ and in the corticotropin-releasing factor precursor⁷. It is interesting that in addition to GRF(1–44)-NH₂ significant amounts of GRF(1–40)-OH and GRF(1–37)-

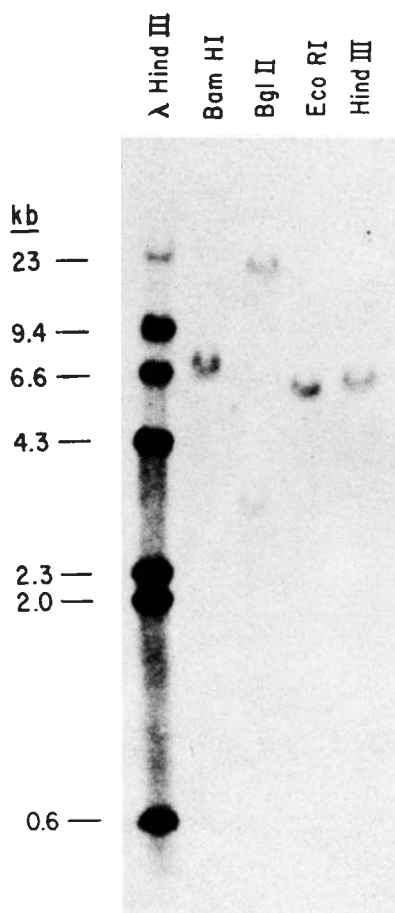


Fig. 4 Southern blot of human genomic DNA probed with the GRF cDNA clone. DNA samples (20 µg) were restricted with the indicated enzymes, electrophoresed on 1% agarose gels and transferred to nitrocellulose by the method of Southern²⁷. The *EcoRI* to *SmaI* fragment from phGRF-54 was nick-translated using [α -³²P]dCTP (NEN) and used as probe. Hybridizations were at 45 °C in 50% formamide, 5×SSPE (1×SSPE = 180 mM NaCl, 10 mM NaH₂PO₄, 1 mM EDTA, pH 7.4), 10% dextran sulphate, and washes were in 2×SSC, 0.1% SDS at 68 °C. The markers (in kilobases) are *HindIII*-cut λ DNA.

OH have been isolated from human tissues^{1,2}. These shorter peptides would also be generated by cleavage before a single arginine residue, and therefore may represent natural variant products. Proteolytic processing of GRF from its precursor would generate two additional peptides, a C-terminal 31-amino acid peptide and an N-terminal peptide of a size dependent on the location of the signal peptide cleavage site (Fig. 3).

GRF contains homology to several other characterized peptides including glucagon, secretin, PHI (peptide with histidine as N-terminus and isoleucine as C-terminus), vasoactive intestinal peptide and gastric inhibitory peptide^{1,2}. We therefore used the GRF cDNA probe to screen Southern blots of human genomic DNA in an effort to identify homologous genes in this family. In moderate-stringency conditions, only one or two bands appear with each restriction enzyme tested (Fig. 4), indicating that the GRF gene is probably unique in humans and is not highly homologous to the genes encoding other related peptides. The failure to identify more than one GRF gene suggests that the cDNA isolated from a pancreatic tumour is the product of the same gene that gives rise to the mRNA in the hypothalamus. In agreement with this notion, Southern blot analysis indicates that no gross rearrangement of the GRF gene has occurred in DNA from the pancreatic tumour from which the GRF cDNA clone was isolated (unpublished results). Since

this technique has limited resolution, it is not yet possible to state whether any genomic alterations can be associated with the ectopic production of GRF by this tumour.

Our results suggest that expression-cloning may provide a powerful approach to the isolation of cDNAs encoding small peptide hormones and neurotransmitters. The availability of a cDNA clone for human GRF will allow us to isolate and characterize the human GRF gene. It should also be useful for the isolation of GRF cDNA or genomic clones from animal species in which it will be possible to study the mechanisms underlying neural and hormonal regulation of this gene. Lastly, these probes may be of value in the diagnosis of human growth disorders.

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Following submission of this manuscript, cloning of a human GRF cDNA was reported by Gubler *et al.*¹⁸.

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Is hypomethylation linked to activation of δ -crystallin genes during lens development?

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Expression of many cell type-specific genes is correlated with a reduced level of cytosine methylation^{1,2} and some results argue that genetic programmes may be activated by a reduction in DNA methylation³. During embryogenesis, however, when many genes are activated in specific cell lineages, it has not been demonstrated that they are hypomethylated prior to their expression. We have examined the timing of hypomethylation and gene activation during embryonic chick lens development

for the two genes encoding δ -crystallin (the major lens-specific protein). We report here that while many of the CCGG sequences analysed become hypomethylated, most do not do so until 2 days after δ -crystallin is first synthesized. However, there is at least one site which is hypomethylated earlier, approximately when transcription is thought to commence. We conclude that hypomethylation in the δ -crystallin genes is probably not a simple process which activates transcription, although early hypomethylation events indicate obvious sites to be examined for a role in gene activation.

We have monitored methylation changes at the sequence CCGG in the δ -crystallin genes of lens ectoderm DNA by treatment with either *MspI* or *HpaII*. Both enzymes cleave DNA at CCGG sequences, but only *MspI* cleaves this sequence if the inner cytosine is methylated, thereby allowing identification of methylated sites by comparing digests from both enzymes⁴. Fragments of the δ -crystallin genes from these digests were identified using a cDNA probe (Fig. 1) in a hybridization procedure modified to detect these genes in small amounts of DNA (Fig. 2).

We first compared methylation levels of the δ -crystallin genes in 13-day embryonic lenses, which synthesize δ -crystallin, and in 13-day headless embryos, which do not synthesize δ -crystallin. *MspI* digests of these two samples were identical (Fig. 2A), although we did find a polymorphism between the two strains of chick embryos used here since one has a 2.3-kilobase (kb) fragment not found in the other (compare emb. 1 with emb. 2 or lens 2 of Fig. 2A). The fragments detected by hybridization with *HpaII* digests of 13-day lens and headless embryo DNA are quite different (Fig. 2A), indicating hypomethylation of lens DNA. Several fragments of lens DNA (4.2, 2.0 and 1.5 kb) hybridize to the cDNA probe which are not found, or are greatly reduced, in headless embryo DNA. A 4.9-kb fragment in headless embryo DNA is greatly diminished in lens DNA, presumably because it has become hypomethylated at an internal CCGG site. While the 4.2-kb and 2.0-kb fragments match *MspI* fragments, the 1.5-kb fragment does not. Mapping experiments indicate that this fragment is probably generated by the adjacent 0.6-kb and 0.8-kb *MspI* fragments in gene 2, which remain uncut by *HpaII* because the CCGG site at their junction remains methylated (data not shown). As the 2.0-kb fragment is from gene 1 (Fig. 1), it appears that there is hypomethylation in both genes. Similar results have been obtained by others⁵.

To determine when these changes first appear during lens development, we have examined lens tissues both before and after the appearance of δ -crystallin⁶ (and δ -crystallin mRNA⁷) at 48–52 h. The earliest sample was from 40-h embryos, when lens ectoderm is not yet differentiated, although probably committed to lens formation⁸. Figure 3 shows the developmental stages used in our experiments and the protein accumulation in

each on a one-dimensional SDS-polyacrylamide gel. We first detect δ -crystallin by this assay at 58 h of development.

HpaII digests of DNA from several stages of lens development (Fig. 2B) show that hypomethylation occurs in both δ -crystallin genes, but, at the sites examined, only after δ -crystallin synthesis has begun. DNA samples from 52-h or 65-h lens ectoderm show none of the four changes in fragments found in 13-day lens. In 96-h lenses the amount of the 4.2-kb fragment increases substantially and the 2.0-kb and 1.5-kb fragments are detectable. The amounts of these fragments continue to increase in 144-h lenses, but are not present to such an extent as in 13-day embryonic lens (Fig. 2A). The decrease in the amount of the 4.9-kb fragment seen in 13-day lens must occur quite late, as it is still prominent even in 144-h samples.

If the δ -crystallin genes were active in a small fraction of cells at 48 or 65 h, and these genes were hypomethylated, we might not have detected this small amount of unmethylated DNA; however, immunofluorescence studies⁹ show that in 65-h lens ectoderm ~75% of the cells are synthesizing δ -crystallin.

The experiments in Fig. 2 were repeated, but DNA was digested with *BamHI* as well as *HpaII*, since this facilitates mapping of DNA fragments, which can be difficult in the partial digests from *HpaII* treatment alone⁴. These double digests allowed us to identify several additional methylated sites. *BamHI* was used here because there is one large *BamHI* fragment in both δ -crystallin genes which hybridizes to our cDNA clone (Fig. 1). Digests of DNA from 13-day lens and headless embryos (Fig. 4A) show the differences at 2.0- and 1.5-kb seen in *HpaII* digests, as expected because these fragments lie within the boundaries defined by the *BamHI* cuts (Fig. 1). We also see a difference at 2.9 kb and a less prominent difference at 2.6 kb resulting from cuts at the left *BamHI* boundary and the first *MspI* site in gene 1 and gene 2, respectively.

HpaII/BamHI digests of DNA from lens ectoderm at different developmental stages show that all of these differences between 13-day embryonic lens and headless embryo δ -crystallin genes occur predominantly after δ -crystallin synthesis begins (Fig. 4B). At the youngest stage (40 h), none of the four fragments (2.9, 2.6, 2.0 and 1.5 kb) was observed. At 52 h, the 2.9-kb fragment was the only fragment detectable. In some preparations of 65-h lens ectoderm, the 1.5-kb fragment appeared faintly. Most of the hypomethylation events do not occur until 96 h, when all four fragments are first seen. The 2.6-kb fragment hybridizes weakly, but is increased in 13-day lens (Fig. 4A).

Evidence for two early hypomethylation events is also seen in *HpaII/BamHI* digests (Fig. 4B). A 10-kb fragment found at early timepoints is probably the 10-kb *BamHI* fragment in gene 1 with the three internal *MspI* sites completely methylated. The amount of this fragment is reduced by 65 h and it is almost

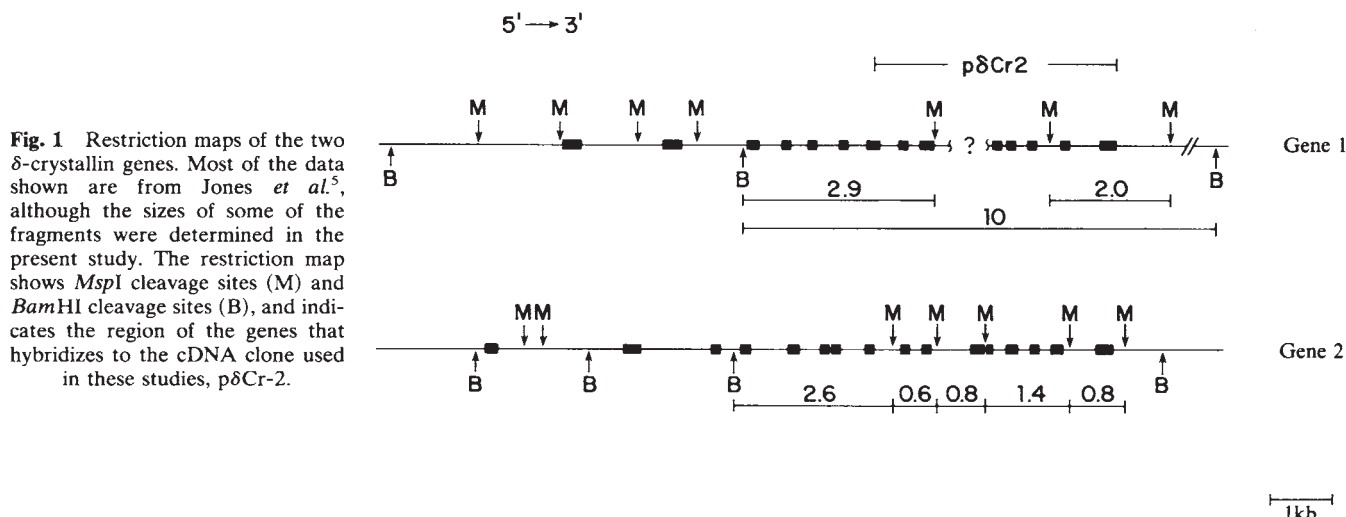


Fig. 1 Restriction maps of the two δ -crystallin genes. Most of the data shown are from Jones *et al.*⁵, although the sizes of some of the fragments were determined in the present study. The restriction map shows *MspI* cleavage sites (M) and *BamHI* cleavage sites (B), and indicates the region of the genes that hybridizes to the cDNA clone used in these studies, p δ Cr-2.

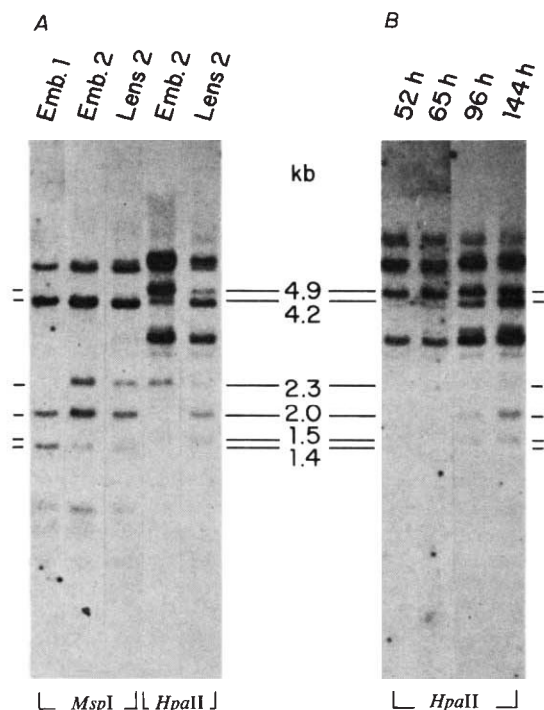


Fig. 2 Changes in the *HpaII* digestion pattern of the δ -crystallin genes in different tissues and in lens ectoderm during development. **A**, DNA samples were taken from 13-day-old headless embryos (Emb.) or 13-day embryonic lens (Lens) and digested with the enzyme indicated at the bottom of the figure. DNA from two different strains of chickens (1, line 22, Spafas Inc., Norwich, Connecticut; 2, Ross-Arbor Acre) shows a polymorphism in the δ -crystallin genes. All of the samples in other experiments were prepared from the second strain. **B**, DNA samples were taken from lens ectoderm at different developmental stages; embryos were incubated for the times indicated and lens tissues corresponding to the stages shown in Fig. 3 were collected. All of these samples were digested with *HpaII*. In this figure, appropriate lanes from different sections of a gel or from different gels have been juxtaposed. The amount of DNA in each lane, specific activity of the hybridization probe, hybridization conditions and length of exposure of autoradiograms were all virtually identical for different experiments, allowing us to compare different experiments in this way. Each timepoint was repeated with at least three different samples to ensure reproducibility.

Methods: DNA was isolated from pooled samples of the appropriate tissue and digested with either *MspI* or *HpaII*. Φ X174 DNA was added as a standard to each digest to monitor complete cleavage. Samples were electrophoresed on 1.2% agarose gels and transferred to nitrocellulose filters¹⁷. Filters were hybridized at 68 °C in aqueous solution¹⁸ with p δ Cr-2 DNA labelled with ³²P-dCTP¹⁹, using some modifications of standard techniques. As we could collect only 1–2 μ g of DNA from several hundred lens ectoderm rudiments at the younger developmental stages, it was imperative to use very high specific activity probes and to minimize background to detect the δ -crystallin genes in these samples. To reduce the background during hybridization, the hybridization solution, containing 10⁸ c.p.m. of labelled p δ Cr-2 DNA, was first reacted with a blank piece of nitrocellulose in a 16-h mock hybridization. The solution was then reboiled to denature the probe and used for hybridization with the experimental filter. This pretreatment greatly reduced the amount of nonspecific binding, which otherwise limits the sensitivity of the hybridization techniques.

completely digested by 144 h, presumably because of progressive hypomethylation of internal CCGG sites. The most dramatic early change seen is the transient appearance of a 5-kb fragment, from 52 h until 96 h, although occasionally it is not seen until the 65-h stage. This fragment is larger than any single *MspI*/*Bam*HI cleavage product and probably results when a fragment is released that still contains at least one methylated CCGG site internally. The disappearance of the 5-kb fragment at 144 h would probably be the result of a later hypomethylation

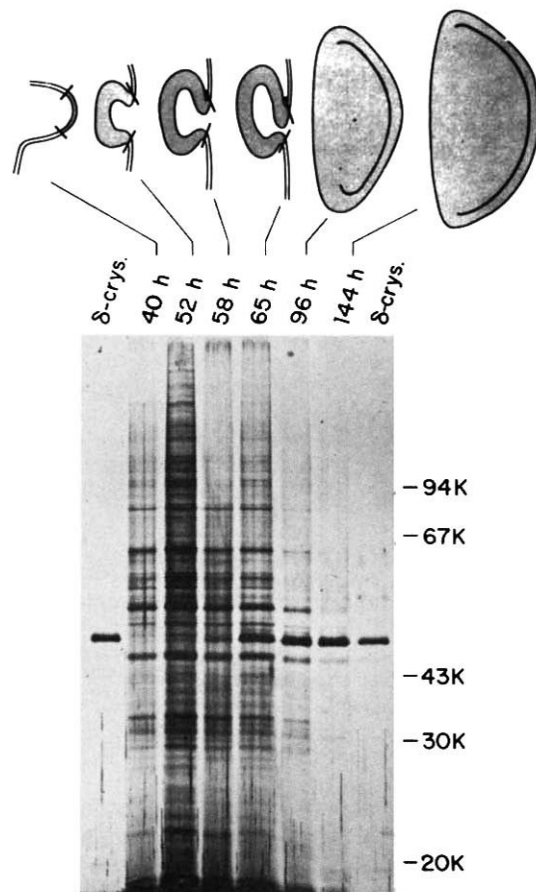


Fig. 3 δ -Crystallin accumulation at different stages of lens development. Embryos were incubated for the number of hours indicated, and from these, lens tissue (or presumptive lens ectoderm) of the exact stage illustrated above each timepoint was selected after dissociation from adjacent tissues by trypsin treatment. There was considerable variation in developmental age in younger embryos and it was essential to pick only carefully staged lens tissues to minimize variability among timepoints. The stippled area in each illustration indicates the region of tissue which was collected. The major proteins from these developmental stages are seen on the SDS-polyacrylamide gel. The lanes labelled δ -crys. were samples of 13-day lens protein, which is almost entirely δ -crystallin.

Methods: Tissues were staged according to O'Rahilly and Meyer²⁰ for 40–96-h samples; after this time embryos were staged according to Hamburger and Hamilton²¹. Our 40-h, 52-h, and 58-h samples corresponded to stages 11, 14 and 15, respectively. The 65-h samples were very late stage 16 or early stage 17. There seemed to be less variability in 96-h and 144-h samples, and we generally took all lenses from embryos of these ages; these were stages 24 and 29, respectively. The actual size difference between younger and older stages is greater than that shown. Samples from each developmental stage were electrophoresed on a 0.75 mm-thick 10% SDS-polyacrylamide gel²² and proteins detected by a silver staining procedure²³.

event at the internal CCGG site(s). This fragment never hybridized to the same extent as some fragments, but since it is transient, large amounts would not be expected to accumulate. We have not yet determined the location of the 5-kb fragment within the δ -crystallin genes.

We were concerned that the 5-kb fragment appearing at 52 h might have resulted from a general hypomethylation event in all cells, as there was some hypomethylation of the δ -crystallin genes even in non-lens tissues at 13 days, compared with early samples of lens ectoderm (Fig. 4). Neither the 5-kb fragment nor any of the other fragments found later, however, were observed in 40-h body ectoderm DNA or 65-h headless embryo DNA (Fig. 4C).

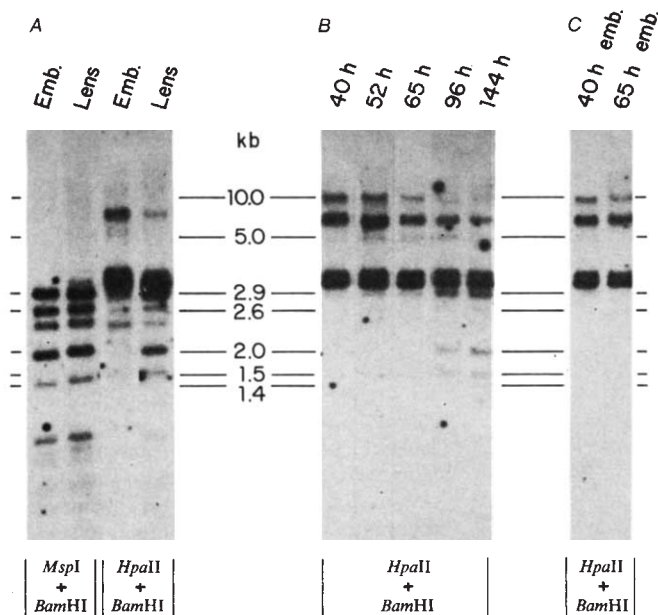


Fig. 4 Changes in the *HpaII*/*Bam*HI digestion pattern of the δ -crystallin genes in different tissues and in lens ectoderm during development. **A**, *HpaII*/*Bam*HI digestion of 13-day headless embryo (Emb.) and lens DHA (Lens) with the enzymes indicated at the bottom of the figure. **B**, *HpaII*/*Bam*HI digests of lens ectoderm DNA from 40–144-h embryos staged as described for Fig. 3. **C**, *HpaII*/*Bam*HI digests of DNA from 40-h body ectoderm and 65-h headless embryos. The procedures in these experiments were identical to those described for Fig. 2, except that simian virus 40 DNA was used as an internal standard to monitor complete cleavage during restriction enzyme digestions.

While the appearance of the 5-kb fragment may be related to gene activation, we cannot be certain that this event precedes transcription because of limitations in staging embryos and because there may be low levels of transcription before this stage. Our assay would not have detected a possible earlier event in the hypomethylation process, the formation of hemimethylated DNA at this site, since *HpaII* does not cleave hemimethylated DNA¹⁰. This could be a functionally important step affecting transcription. As *HpaII* analysis only monitors a small fraction of the possible methylated sites (and in this study only those *HpaII* sites in the 3' region of the genes), there could also be other methylation changes which occur even earlier than that observed at 52 h.

Several studies argue that hypomethylation does not occur when transcription begins^{11,12}, or that it is not sufficient for transcription¹³; however, our results indicate how important it may be to assay many methylation changes during gene activation to observe a few sites which are tightly coupled to the onset of transcription. Only by a developmental profile can one identify transient products, such as the 5-kb fragment we observed. Unfortunately, this is not a simple procedure in most embryonic systems. The only other study of this kind¹⁴ showed that in the red blood cell lineage there is no hypomethylation of the globin genes 12–15 h before globin is synthesized. Here, however, because it was impossible to isolate a pure population of red blood cell precursors during the 12 h before globin gene activation, the exact timing of hypomethylation could not be determined.

As most of the methylation changes we see follow the onset of transcription, they cannot have a causal role in gene activation; it is possible that they affect transcriptional efficiency or stabilize the transcriptionally active state. If these sites were hemimethylated earlier, they might have a role in gene activation; however, this seems unlikely as the 48-h gap between first δ -crystallin synthesis and the appearance of these hypomethylated sites far exceeds the one cell cycle (9 h¹⁵) expected to yield hypomethylated DNA from hemimethylated DNA.

Several of the methylation changes which follow δ -crystallin gene activation (Fig. 4B) are also seen in non-lens tissues from older embryos (Fig. 4A; ref. 16), further suggesting that they are not involved in transcriptional regulation. Although the δ -crystallin genes are transcribed at extremely low levels in some non-lens tissues¹⁶, other control mechanisms, possibly including other methylation changes, must also be important in modulating the level of δ -crystallin transcripts in these tissues.

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Errata

IN the letter 'Viability of λ phages carrying a perfect palindrome in the absence of recombination nucleases' by D. R. Leach and F. W. Stahl, *Nature* **305**, 448–451 (1983), the two figures were transposed. Figure 2 should show the micrograph of Red⁺ DNA (B) and gels of restriction digests (A).

IN the letter 'Evidence for opiate receptors on pituicytes' by S. L. Lightman *et al.*, *Nature* **305**, 235–237 (1983), the plates for Figs 1 and 2 on page 236 have been transposed.

IN the letter 'Discovery of a 6.1-ms binary pulsar PSR1953+29' by V. Boriakoff *et al.*, *Nature* **304**, 417–419 (1983), in Table 1 $\langle n_c \rangle$ should read $\langle n_e \rangle$ and the top line in the mass function in Table 1 should read $m_c^3 \sin^3(i)$.

Variations in magnetization intensity of ocean floor basalts

A REPORT by Bleil and Petersen¹ analyses data collected on 3,500 basalt samples retrieved from the ocean floor. The authors could account for the observed patterns of marine magnetic anomalies merely on the basis of a simple mechanism of low-temperature oxidation of titanomagnetite grains in basalts and consequent changes in the intensity of magnetization. The facts and figures given in the paper appear convincing if taken at their face value, as does any cross-checking if limited to the references cited therein. However, for a critical reader who is also a researcher in rock magnetism, the presentation betrays a one-sided and oversimplified approach. I wish to substantiate this remark by the following points.

(1) Although the average bulk composition of the magnetic grains of oceanic basalts may correspond to that given by the authors, it is over-optimistic to consider that all marine basalts causing the magnetic anomalies consist of a single specific member of the titanomagnetite solid solution series and its oxidation products. To cite one extreme example, Murthy *et al.*² have shown that a peridotite sample from Leg 37 contained almost pure magnetite as inferred from the magnetic properties.

(2) The oxidation parameter z , which the authors considered as the key factor, is not a directly measurable quantity but is derived from two others, one often being the Curie temperature which itself cannot be determined for basalts uniquely³. Moreover, basalts often indicate more than one Curie temperature². Although z is a highly favoured parameter among a significant school of rock magnetists, its uniqueness and significance as applicable to basalts become questionable if only this school gives a minimum consideration to the facts mentioned below.

(3) Studies on the magnetic domain state aspects of titanomagnetites^{4,5} indicated quite a complex behaviour for these minerals and hence the compositional features of the grains derivable from chemical and X-ray analyses on the one hand and from magnetic techniques on the other, could differ widely. Certain magnetic properties like Rayleigh loops (hysteresis loops in a field of 10 Oe) shown by basalts could be explained only by invoking superparamagnetic behaviour for some of the magnetic grains present in the samples⁶. The values of intensity of natural remanent magnetization and susceptibility will be highly sensitive to temperature if the basalts concerned show Rayleigh loops, and the issues raised by Deutsch

and Pätzold⁷ cannot be ignored in a model for magnetic anomaly interpretation.

In September 1979, the Rayleigh loops of several oceanic basalt samples from Petersen's collection were photographed in his own laboratory when I was visiting him and we discussed their implications at that time. It is surprising that the impact of such observable properties were not mentioned in their paper¹.

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BLEIL AND PETERSEN REPLY—We agree with Radhakrishnamurty that our explanation of the temporal variation of magnetization intensity of oceanic basalts¹ is necessarily a simplification of the mechanism underlying this phenomenon. However, we cannot agree with particular points which he raised against our arguments.

(1) Our data refer to the uppermost 500–1,000 m of oceanic basalt, which are directly accessible due to the drillings of the Deep Sea Drilling Project. Analyses of the magnetic mineral components contained in these rocks (predominantly mid-ocean ridge basalt (MORB)) show a surprising uniformity in composition, in correspondence to the uniformity of the overall geochemistry of the rocks. We are well aware that at greater depths, below seismic layer 2A, the magnetic mineral component is likely to be different from that described in our paper. An example of this kind is the peridotite sample cited by Radhakrishnamurty. A possible contribution of such deep-seated sources to the marine magnetic anomaly pattern will consequently show a different temporal magnetization variation, compared with that proposed in our model. It must be kept in mind, however, that the main portion of magnetic sources is located within the uppermost 500–1,000 m of the oceanic crust² which is MORB of essentially uniform composition.

(2) In evaluating age trends of the magnetic properties of oceanic basalts, it is not a prime question which parameter is chosen as reference, as long as this para-

meter can be defined uniquely. In our case, this parameter was the Curie temperature, which was transformed to the titanomagnetite oxidation parameter z using the relationship derived by Petersen *et al.*³. The latter relationship is based on chemical analysis of titanomagnetite extracted from ocean floor basalts. It is not correct to say that Curie temperatures of oceanic basalts cannot be determined uniquely. For such a statement the number of ambiguous Curie temperature analyses has to be seen against the total number of Curie temperature analyses carried out on ocean floor basalts, most of which are unambiguous (see, for example, refs 4–7).

(3) Radhakrishnamurty contends in his third point that the domain state of the magnetic minerals rather than their ferromagnetic structure—as proposed by us—is the cause for the described temporal variation of magnetization intensity. That could be due either to superparamagnetism that develops in the magnetic minerals in the course of seafloor alteration (as proposed originally by Butler⁸), or to an effect of enhanced induced magnetization at slightly elevated temperatures by the Hopkinson effect, as proposed by Deutsch and Pätzold⁹. However, a comparison of our Figs 2, 3 and 5 (ref. 1) shows that for a rock of a certain oxidation state, the magnetization minimum will be the same for both remanent and saturation magnetization. This observation strongly contradicts Radhakrishnamurty's suggestion. If a systematic variation of the domain state of the magnetic minerals were responsible for the observed trend, it should not be seen in the saturation magnetization.

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Phoney doctorate?

Fred Dainton

How the PhD came to Britain: A Century of Struggle for Postgraduate Education.

By Renate Simpson.

Society for Research into Higher Education, Guildford: 1983. Pp.205. £9.75.

By international standards Britain has always lagged behind in the provision of higher education. By the time, just over 150 years ago, Babbage published his tract on the decline of science and the British Association for the Advancement of Science emerged, with a little help from academics from the York Philosophical Society, England had only two universities, Ireland one and Scotland four; all founded several centuries earlier.

Oxford, Cambridge and Trinity College, Dublin saw their prime role as the education of those children of the ruling class who were destined neither to administer the estates they would inherit nor to serve in the armed forces but to join the learned professions of the law, the Church and medicine. Though the award of an MA degree was the necessary qualification for teaching within a university it did not grant the holder a licence to practise his chosen profession outside it. Moreover, the collegiate system separated the tutors from the professors, often placing a higher value on the former.

The Scottish university tradition was much more one of practical service to the community; professors were esteemed for what they knew and could teach, access was more open and whilst, half a century earlier, Gibbon was justly complaining about Oxford, Adam Smith was writing his *Wealth of Nations* in Glasgow and the physician, Joseph Black, having done pioneering research in Glasgow on *magnesia alba*, 'fixed air', latent and specific heats was, as Professor in Edinburgh, stimulating students' minds through his splendid lectures on chemistry.

Systematic enquiry into knowledge, which we now call research, was a late-comer to the English university scene. Public recognition of research as a proper, indeed necessary function of universities came first in Germany where the Prussian Minister of Culture and Education, von Humboldt, who created the University of Berlin in 1812, saw clearly that teaching at the highest levels must go hand-in-hand with research and that the professors must have solitude and time to think (*Einsamkeit*) and complete freedom (*Freiheit*) about what should engage their minds and what they should teach. Fitness to research was recognized by the establishment of the D.Phil degree obtained by thesis and examination, and for which high level courses of instruction had to be attended, seminars were instituted and became common, and as a

corollary, grades of academic posts were established to which appointments were made by the state on the recommendation of properly constituted competent knowledgeable committees.

The results were that the professor in his specialized institute was omnipotent, that Germany attracted many foreign aspirants to scholarship and to science who wished to secure a degree. Many of these on returning home applied pressure to initiate research in the universities of their native countries, pressure augmented in the USA by German migrants and simultaneously complemented by the ideal of universities as social utilities which was carried to that country by Scottish migrants.

Britain, pre-occupied with its imperial, commercial and industrial growth, only belatedly awoke to the deficiencies of its universities in number and in research, despite the strictures of many able critics like Mark Pattison. The movement to create university colleges in London and the great manufacturing and mercantile cities, often later to be linked with independent medical schools and become the 'Old civic' universities of today, addressed the problem of number but with the exception of the MD the only doctorates which were offered required upwards of ten years of work and a very substantial amount of original and independent publication. In no sense did this contribute to the production of cadres of young people trained in systematic enquiry. True, Masters degrees in arts and science, obtainable by research were introduced but these were regarded as inferior in quality to the German PhD.

The second decade of the twentieth century was crucial for the United Kingdom in these matters. Far-sighted men like R.B. Haldane, C.C. Addison and H.A.L. Fisher saw the necessity for state-supported research and universities. Haldane (who had been educated partly in Göttingen) and Addison correctly differentiated between research for 'general purposes' and that for 'departmental use' and they actively promoted the concept of the research councils and were influential in establishing the Medical Research Council and the Department of Scientific and Industrial Research. Haldane, as a member and chairman of one of the precursors of the University Grants Committee had been a major influence in devising criteria for the disbursement of government funds to universities. Fisher, a former Vice-Chancellor of Sheffield Uni-

versity (in which Addison had earlier graduated and been the first full-time Professor of Anatomy before embarking on a distinguished political career) was President of the Board of Education and gave powerful support to the formation of the Committee of Vice Chancellors and Principals. He encouraged their co-operation in post-graduate work and was a proponent of higher degrees for foreign post-graduate students. These would otherwise depart without any formal university recognition of their work other than some certificate of diligent study. Paradoxically the years 1918 and 1919 saw not only the onset of the decline of British Imperial power but also the dawn of a new era for British Universities with the formation of the Committee of Vice Chancellors and Principals, its affirmation of intent to establish the PhD degree and the issue of a Treasury minute giving the terms of reference to the University Grants Committee.

Renate Simpson tells this story in a new and engaging way. New because she had access to Government papers which only recently became available under the "50 year rule" and, lacking those records of what is now the Association of Commonwealth Universities which were destroyed during the Second World War, she has painstakingly searched many university archives; engaging because the style and the comments suggests a quizzical, sceptical, outsider looking in and is all the better for that. I warmly recommend the book to all interested in the history of higher education in the UK; it is of a higher standard than other books published in this series which I have read.

What the introduction of the PhD did was to institutionalize academic research in Britain, to create an apprenticeship and certification system, which has led to the growth of research constituencies in universities. For at least 30 years the effects can be described as beneficial but in the last 30 years the defects of the PhD system have not gone unnoticed.

Who will now tackle problems such as the balance between course work and research, the excessive 'academicism' of PhDs in engineering and technology, the too frequent laxity of supervisors and soft-heartedness of examiners, the failure of students, especially in the social sciences, to complete the course in the prescribed time if at all and kindred issues? Will the reform come from within, as it should, or must external forces impose it? And when the changes do come about will their chronicler be as conscientious, perspicacious, critical and balanced as Renate Simpson? The answers to these and related questions are in the future but purposeful search for them must begin now if the PhD is to retain esteem in future years. □

Sir Frederick Dainton is Chancellor of the University of Sheffield.

A multitude of probes

T.S. West

Soil Analysis: Instrumental Techniques and Related Procedures.

Edited by Keith A. Smith.

Marcel Dekker: 1983.

Pp.562. SFr.198, \$69.50.

THE revolution that has occurred in analytical technology during the past 20 years and the varying impact this has had in different areas of application are given as the reasons for the publication of *Soil Analysis: Instrumental Techniques and Related Procedures*. The text is designed to bridge the gap between the many specialist monographs on instrumental techniques that crowd the bookshelves and those on traditional methods of analysis. The authors, drawn mainly from agricultural research laboratories, aim to show how modern physicochemical techniques may be used to tackle the problems of soil analysis on a routine basis.

Each technique is treated monographically on the basis of principles, equipment, application to soil science and, usually, conclusions. There is, in most cases, a generous list of references. The book covers atomic absorption and flame emission spectrometry, ion selective electrode potentiometry, automated instrumentation for (total) carbon, nitrogen and sulphur in soils, X-ray fluorescence spectrometry, general radioisotope tech-

niques, instrumental neutron activation analysis, determination of nitrogen isotopes by optical emission spectrometry and mass spectrometry, gas chromatography analysis of soil atmospheres and pesticide determinations by gas and high pressure liquid chromatographies.

Most of the treatments are excellent in concept and execution and reveal that soil scientists, as typified by the authors, are very much at the forefront of modern analytical technology. The capabilities and limitations of the individual techniques are also made clear and there are many fine diagrams and graphical presentations of results. The tabular summaries of data are most useful and attractively set out.

This is a fine volume which gathers much useful information for soil scientists together between two book covers, but I hope Dr. Smith and his colleagues may be persuaded on a future occasion to give the book more focus by saying at the outset why all these analyses are necessary, what their significance is to plant and animal life as well as to soils themselves and perhaps, at the end of the book, compare methods and assess when one might, for example, use an electrode rather than atomic absorption to determine some trace element.

The book would also benefit from some discussion of concepts of 'plant availability', soil extractants, inter-element synergic effects, etc., near the beginning, thus to make the novice, as well as even the expert on occasion, aware of what he is looking for as he goes through the book. □

Professor T.S. West is Director of the Macaulay Institute for Soil Research, Aberdeen.

Getting down to the biology business

M. Cole and R. Smither

Legal Protection for Microbiological and Genetic Engineering Inventions.

By R. Saliwanchik.

Addison-Wesley: 1983.

Pp.300. \$34.95, £26.20.

Biochemistry and Genetic Regulation of Commercially Important Antibiotics.

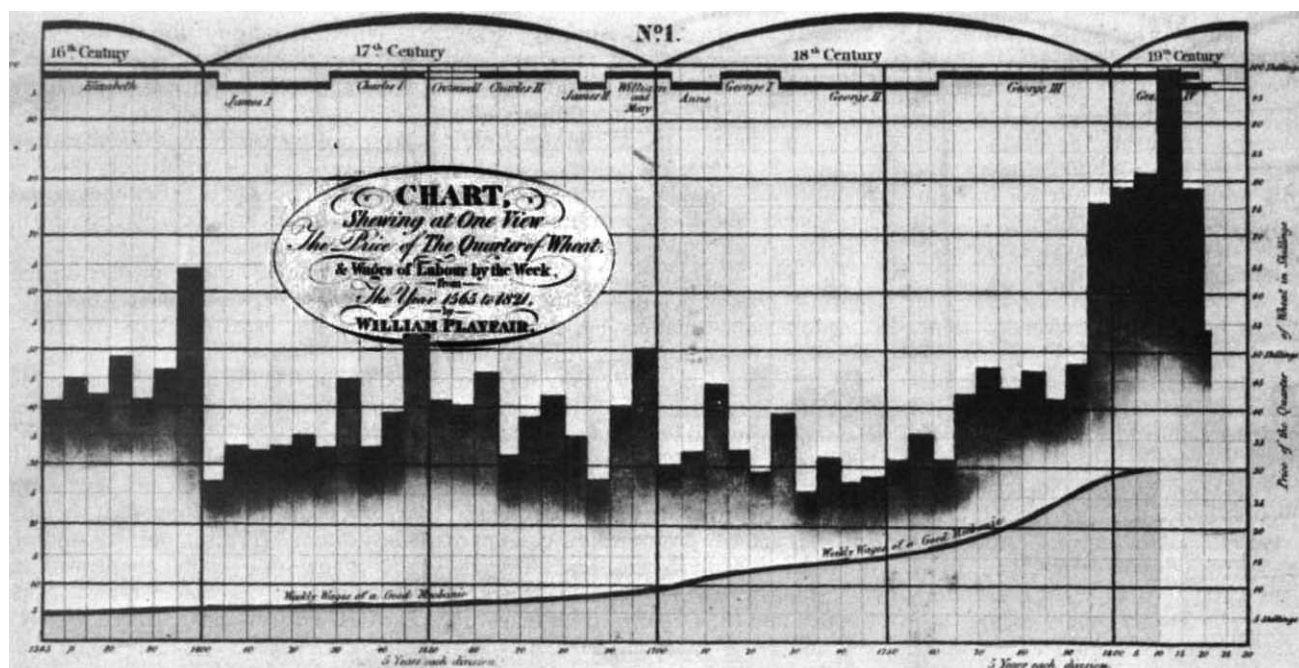
Edited by L. Vining.

Addison-Wesley: 1983.

Pp.375. \$39.95, £33.95.

IT MIGHT seem odd that one of the first two books in this promising series on various aspects of biotechnology should deal with patent aspects, but one of the most important driving forces for research in this area is the possibility of commercial exploitation. The second book, more in line with expectations, deals with one of the foundation stones of the subject, namely antibiotics.

The patenting of microbiological inventions has always had its complications but in the last decade, the continuous development of genetic engineering has moved the science of microbiology into a new era and the patent systems of the industrialized countries of the world are having to embrace these new techniques. Roman Saliwanchik's book is a detailed study of patent law relating to micro-



Pioneering the display of data — the chart shows both the price of wheat in the UK over 250 years and the wages of a good mechanic such as a smith, mason or carpenter, over the same period. It also shows the reigns of kings and queens. It was drawn early in the nineteenth century and is taken from *The Visual Display of Quantitative Information* by Edward R. Tufte (Graphics Press), which will be reviewed in next week's *Autumn Books Supplement*.

biological inventions in the United States, although there are two chapters devoted to the corresponding position in certain other countries.

Saliwanchik has drawn very heavily upon many practical examples of US patent prosecution in the microbiological field and the relevant case law. The necessary complexity and detail of these examples as well as the lengthy recitation of the procedural requirements of the US patent system may well have little appeal to the majority of research scientists. They will continue to leave the legal protection of their inventions to the professional patent advisors and in-house patent departments for whom this book will be most useful. It will further be of value to non-US patent practitioners wishing to gain an insight into this specialist field.

The chapter on trade secret protection is well presented and most timely. It is the onerous requirement of most patent offices of the world (the US is the major exception) that where a patent relies on the use of a new strain of micro-organism, the strain must be on open deposit in a recognized culture collection at the time of first publication of the patent application. This may lead to an increase in keeping microbiological inventions as trade secrets.

The requirements of description and enabling disclosure for microbiological patents go beyond what is called for in other branches of technology and accordingly, for the microbiological inventor, the patenting route is often less attractive than for other inventors. Saliwanchik's book serves a most useful purpose in highlighting such problems and bringing them to the attention of all workers in this field.

As the subject of the second book, antibiotics, broadens by the continuous addition of newly discovered substances, so the investigations on the main classes of established economically valuable substances grow in depth. The book succinctly illustrates the ways in which advances in methods of studying genetic regulation, biosynthetic intermediates and cell free enzymic conversions have added enormously to the understanding of how microbial metabolites are made. However, the extent of what we do not know is also clear and the book will whet the appetite of anybody looking for new research topics. Those who choose to work in this area could find themselves making commercially interesting observations either in the form of new routes to the synthesis of economically important antibiotics or the discovery of new compounds by the application of the newer methods of hybrid biosynthesis.

The editor Leo Vining has succeeded in bringing together the contributions of many well known people, to the subject of antibiotic biosynthesis. Although the title refers to commercially important antibiotics, it deals with whole classes of compounds and is far more interesting

because of this. It is also up to date. Although it does not deal with the mode of action of antibiotics most chapters give a brief indication of which compounds are commercially important and why.

The chapters on the genetics of streptomycetes and fungi come at the beginning of the book — a sign of the times. Here you will find discussion of new systems for genetic analysis and the role of plasmids in antibiotic synthesis. Arnold Demain and his colleagues, clearly describe metabolic control and smooth the way into the chapters by other authors on specific classes of compounds. These include β -lactams, peptides, peptidolactones (actinomycins etc.), macrolides, polyenes, ansamycins, tetracyclines, anthracyclines, chloramphenicol, aminocyclitol glycosides and the lincomycin-anthramycin group.

Those interested in enzymes will appreciate the discussion of multienzyme

complexes to be found in the chapter on the synthesis of peptide antibiotics. Three such complexes are involved in the construction of the cyclic decapeptide, tyrocidine. They catalyze ten amino acid activations, two epimerisations, nine specific elongations and one cyclization. How about getting that to work in a multienzyme reactor! This is just one of the many fascinating aspects of antibiotic biosynthesis which you will find by browsing through this thoroughly recommended and well presented book.

If the other books to be published match up to these two volumes we will have a very valuable series. □

Martin Cole is a Senior Research Associate in Beecham Pharmaceuticals Research Division, Yew Tree Bottom Road, Epsom, KT18 5XQ and Ron Smither is Patent and Trademarks Controller for Beecham Group, Great West Road, Brentford, UK.

Boundary effects

R.J. Bell

Surface Polaritons: Electromagnetic Waves at Surfaces and Interfaces.

Edited by V.M. Agranovich and D.L. Mills.

North-Holland: 1983. Pp. 717. Dfl. 345, \$160.50.

SOLUTIONS to Maxwell's equations, which are bound to the interface between two media, have been known for a long time — particularly to radio engineers. In the last 15 years physicists have discovered their usefulness for studying interfaces and named them surface polaritons or surface electromagnetic waves. They have proven to be especially useful at metal-dielectric interfaces because such surface excitations exist from very low frequencies all the way into the ultra-violet. As a field of study, surface polaritons has matured from the review article stage to the review volume stage. It has, as of yet, provided no standard analytical technique, although a number of techniques are promised with further efforts.

This volume intends to provide a summary of recent work in surface polaritons in sufficient depth to serve the worker in the area and provide a reader with a background in solid state physics an intelligible introduction. Within a somewhat restricted definition of the area, it succeeds fairly well. Chapters cover surface polaritons on dielectrics, semiconductors, metals and metal-liquid interfaces. Later chapters cover the scattering of surface plasmons by surface roughness or by fluctuations in the dielectric function near a phase transition point, and non-linear interactions such as the Raman effect and second harmonic generation. Excluded are surface polaritons on non-planar interfaces and magnetic crystals.

Because this book consists of chapters by different authors, it does suffer some repetition of introductory material but less than is often found in such collectively-authored volumes and with the partial compensation that the chapters, in general, can be read independently. Most of the articles appear to have been finished in the latter part of 1980, although there are occasional references to 1981 publications.

Of particular note are the two articles on the interaction of surface polaritons with surface roughness, one by Heinz Raether and the other by Alexei Maradudin. This work is of interest to those dealing with the optical properties of rough surfaces and with the surface-enhanced Raman effect. The several articles on Raman scattering and other non-linear interactions summarize an area that will surely be productive in the future.

Recently, another book of similar intent has appeared, *Electromagnetic Surface Modes* edited by A.D. Boardman (Wiley-Interscience, 1982). Also a collection of articles by various authors, it takes a broader point of view and includes surface polaritons on non-planar interfaces and magnetic crystals. There is surprisingly little overlap between the two books, which between them, include articles by the majority of the workers in the field. For the solid state physicist interested in using surface polaritons to measure the optical properties of interfaces, the book under review is a better choice than the Boardman volume, but both would be needed to obtain a comprehensive view of what was known about surface polaritons by 1981.

This volume provides a worthwhile summary of surface polaritons for the physicist and chemist interested in the optical properties of interfaces and adsorbed species at interfaces. □

R.J. Bell is a Professor of Physics at the University of Missouri-Rolla.

Predicting the past

Marie Jahoda

The Human Legacy.

By Leon Festinger.

Columbia University Press: 1983. Pp.184.
\$19.95.

ONLY creationists any longer deny that man and monkey have evolved from the same distant ancestor. What happened so to enlarge the scope and complexity of the human mind that it produced the miracles of modern technology, art and science as well as the confusion of the social order?

After an outstanding career of forty years as a psychologist, Leon Festinger became doubtful about the significance of the questions experimental psychologists ask and has turned to archaeology in search of some clues to the answer. Having immersed himself for four years in the archaeological literature and observed some fieldwork he now presents in this book an overview of mankind's development, spanning a period from more than two and a half million years ago to the beginning of written records at about 3,000 BC. Those who know him from his previous incarnation will recognize some continuities: Festinger writes clearly, he is provocative, knowledgeable, imaginative, sometimes opinionated, sometimes wrong, but never boring (at least hardly ever).

The book is a mixture between what is known about prehistory and some speculative guesses where archaeological evidence by its very nature cannot provide answers. Throughout, it faithfully conveys the marvellous, detective-like ingenuity of archaeological reasoning from the discovery and dating of skeletons, isolated bones and tools to aspects of prehistoric ways of life. There are two parts. The first deals with biological evolution. Here the major event was the transition to bipedalism. But Festinger quotes Washburn's convincing arguments that bipedalism *by itself* could have been a handicap, not an adaptive development, in terms of limiting the speed of locomotion for hunting or being hunted and of greater vulnerability since any fracture would be fatal to a bipede. Only the simultaneous development of the brain and the neural system permitted the creative use of the arms freed from the task of locomotion.

This development of the mind also led to the emergence of language. From here on biological changes are minimal and the emphasis throughout the following pages is on man's creativity and capacity to innovate in efforts to control the environment. Evidence suggests that about 500,000 BP fire was under control to keep out the cold and somewhat later, covered shelters existed to protect people against wind and rain.

In the longer second part of the book Festinger deals with biologically modern

man whose artifacts can be dated to 20–30,000 yr BP. The description of the transition from the earliest human beings to modern man, the development of tools, of sedentary living which is now thought to have preceded the development of agriculture, of irrigation, of war and of slavery makes fascinating though frustrating reading. So much remains unknowable, so much speculative. Archaeological evidence from pre-literate societies compels concentration on technology but Festinger goes too far towards a more general technological determinism when he describes religion as a technology. Aware that he is stretching the term beyond its customary limits, he tries to distinguish religious from natural technologies by arguing that whereas the latter were universal skills 'religious technology' was in the hands of specialists. Yet this cannot, by his own testimony, be a crucial distinction, for he previously documents the existence of specialists in natural technologies, which has been ingeniously inferred from the uniformly high standard of tools found at single locations.

Throughout the book the dominant mood is a cheerful pessimism: man's inven-

tiveness has indeed found technological solutions for one major problem after the other only to discover that every problem solved gave rise as an unanticipated consequence to new ones. In the last chapter where Festinger reflects on the present the cheerfulness disappears and there remains a bleak pessimism. The only hope is that "... in five or ten or twenty thousand years a new species of human beings, more able than we, will be around." This resignation stems from some rather simple diagnoses of our immediate social problems such as "inefficiency, featherbedding, strikes, seniority systems".

One could list more serious problems, indeed he does, and even imagine possible solutions not necessarily of a technological kind. But to long for a dim future in which all problems are solved without creating new ones is to long for Nirvana. The essence of being alive is to face challenges and to act. Without this last chapter the book would be a stimulating introduction to some aspects of our prehistoric past. □

Marie Jahoda is Professor Emeritus of Social Psychology and Consultant to the Science Policy Research Unit, University of Sussex.

Building up power

M.C.W. Evans

Photosynthesis. Vol.1 Energy Conversion by Plants and Bacteria. Vol.2 Development, Carbon Metabolism, and Plant Productivity.

Edited by Govindjee.

Academic: 1983. Vol.1 Pp.796. \$79, £52; Vol. 2 Pp.580. \$59, £39.

PHOTOSYNTHESIS, the most important of biological processes, is the subject of a huge research effort involving a wide range of disciplines. Collating and presenting this research in a readily accessible form is, to say the least, a difficult undertaking.

Over the years Govindjee has written and edited a number of books with this objective. These two volumes represent his most ambitious attempt to date. They cover the full range of photosynthesis from the picosecond time scale of the primary photochemistry, through the slowing series of electron transport, ATP synthesis and carbon fixation, to the physiological and ecological problems of productivity and global photosynthetic yields. There is even room for the editor's personal interest in the effects of carbon on the primary reactions and for the heresy that bicarbonate is the source of oxygen in photosynthesis.

To achieve this wide-ranging coverage, Govindjee has assembled a distinguished cast of contributors, and to obtain balance between studies on plants and on bacteria, he has induced some improbable author combinations. The result is that virtually every chapter is of a high standard. Only the

molecular biologists have failed to produce a synthesis, apparently falling out after writing a joint introduction.

Each of the books has an introductory chapter, intended to allow readers from other fields to understand the basis of the subject, the important advances and the salient problems remaining. These are followed by the specialist chapters grouped into subject areas. The first volume covers the membrane-dependent processes, the initial photochemistry, electron transport and ATP synthesis; the second, the "dark reactions" of carbon dioxide fixation, plant growth and productivity.

The first volume suffers rather badly from the common deficiency of multi-author books, with considerable overlap between the chapters, and even duplication of figures and tables in some places. In Vol.2 this has been avoided on the whole, though a major omission is an account of bacterial and algal ecology and productivity.

Although the two-volume work can be criticized on a number of points, there is little doubt that it will be the basic reference book on photosynthesis for a number of years. It has the advantage of dealing with both bacteria and plants and of providing chapters on all aspects of photosynthesis. Although specialists will find that details are already out of date, it provides a good record of the main advances of the past ten years. As such it should stimulate the exchange of ideas between the different disciplines involved, and will be an excellent source for both advanced teaching and research. □

M.C.W. Evans is Professor of Plant Chemistry at University College London.

American Society for Neuroscience

To mark the meeting of the American Society for Neuroscience, to be held in Boston from 7 to 10 November, this week's feature covers products for the neurobiology laboratory.

Microscopes

● A new linear, random access automatic stainer for use in histology and cytology has been produced by Shandon Southern. The Linistain GLX is based on the design principles of the SKI Honeywell stainer — a method for providing consistent uniform staining of paraffin-embedded sections on microscope slides. The design includes a ductless fume extraction unit which automatically removes xylene fumes from the loading and unloading areas. The fume extraction unit uses activated charcoal filter cartridges of a standard type used on the Shandon fume guard which can be slotted in and replaced. The random access and continuous operation of the Linistain GLX gives 28 positions for stains, solvents or other solutions. Each slide is fitted in a plastic slide clip which slots onto a constant speed conveyor chain. A heated loading area is fitted for de-waxing and drying specimens. A continuous water wash is selectable at all positions with an infinitely variable flow adjustment to suit specific requirements. Special supports are provided to ensure efficient draining of waste water. The Linistain GLX will handle up to 6 slides per minute and the staining and drying cycle for one routine slide takes approximately 20 minutes. Staining density can be controlled by varying the concentration of stains or by changing the immersion time.

Circle No. 100 on Reader Service Card.

● Leitz has produced a new inverted microscope. The most interesting feature of the Leitz Labovert is that it sits upright. This means that the controls are not behind the microscope's stand or up in the air, as they are with conventional inverted microscopes. Instead, the Labovert's controls are in the same place as on a conventional ergonomically-designed microscope and its low-positioned object stage gives improved access to samples. The microscope has a 6 V, 20 W quartz halogen lamp for standard uses, but a 12 V, 50 W lamp can be fitted if required.

Circle No. 101 on Reader Service Card.

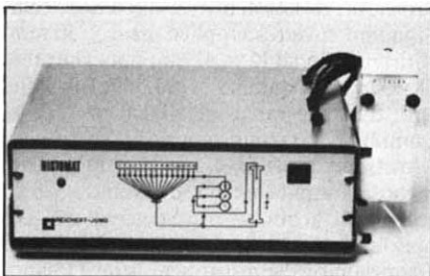
These notes are based on information provided by the manufacturers. For further details circle the appropriate numbers on the Reader Service Card bound inside the journal.

● The lowest continuous range of kV on Philips SEM 505 scanning electron microscope has been extended from the previous 1 kV. Reducing the operational voltage of operational voltage of 200 V enables users to obtain micrographs in the region below 1 kV. Reducing the operational voltage of the scanning electron microscope helps overcome the problems associated with charging the specimen when poorly conducting materials are viewed. This is useful in applications such as in the semiconductor industry, forensic science, biology and ceramics where it is essential to view a sample without applying any conductive coating. The range of accelerating voltage over which charging does not occur depends on the material, the scan speed and the beam current so that the system used should be flexible. In the Philips SEM 505, the operating conditions can be controlled by continuously variable high tension voltage choice of scan speeds (television-rate scan plus 25 combinations of line times and lines per frame for viewing and photography) and by choice of emitter.

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The SEM 505 microscope from Philips



The Histomat and gridstainer from Reichert-Jung

● The automatic production of contrasting ultra-thin sections for electron microscopy is now possible with a new computer-controlled gridstainer which can be attached to the Reichert-Jung Histomat. With the gridstainer the Histomat can accept up to 42 grids in one cycle.

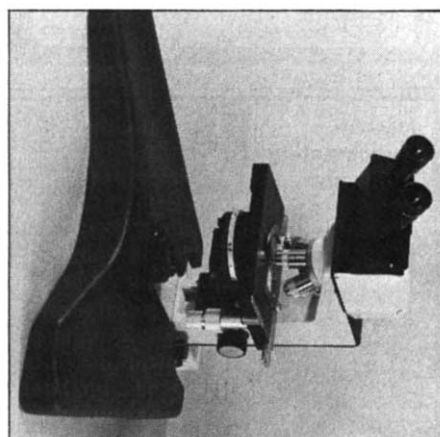
Circle No. 103 on Reader Service Card.

● A removable arm rest which quickly slides over the foot of the microscope has been developed by Leitz Wild Heerbrugg. The soft exterior provides support for extended routine observations.

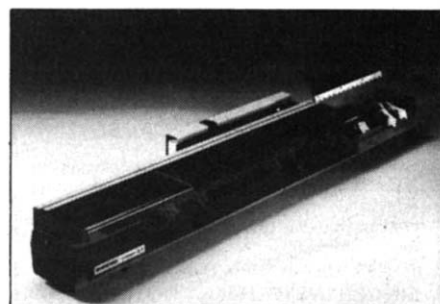
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● The new Histokinette 2000 from Reichert-Jung incorporates twelve processing stations, a programmable timer and an electromechanical transfer and agitation system. The twelve beakers are on a large circular platform and the process cycle is controlled by programmable timer. Both wax baths are thermostatically controlled with overheat and underheat cutouts. Histokinette is suitable for all routine tissue preparations.

Circle No. 121 on Reader Service Card.



The microscope arm rest from Leitz/Wild Heerbrugg



Shandon Southern Products' Linistain GLX

Equipment for neuroscience

● A new micromanipulator controller is now available from Medical Systems. This unit can control two micromanipulators independently. It can position two micro-electrodes to penetrate either the same cell or two different cells in close proximity, and can establish precise bursts of motion to generate stabbing actions which minimize dimpling and assist cell penetration. *Circle No. 105 on Reader Service Card.*

● A new range of immunocytochemistry (ICC) kits is being produced by Metachem Diagnostics. The kits contain all reagents and antisera needed to complete peroxidase-anti-peroxidase immunostaining for a given antigen. Kits are available for 17 peptides including the endorphins, bombesin and somatostatin. *Circle No. 106 on Reader Service Card.*

● The latest addition to the Gamma-B range of iodinated tracers is ^{125}I Tyr¹-somatostatin, now available from RIA (UK). This ^{125}I Tyr¹-somatostatin has a specific activity of 1,000 μCi per μg (37 MBq per μg) and is supplied lyophilized in 5-, 10-, 20- and 40- μCi quantities. *Circle No. 107 on Reader Service Card.*

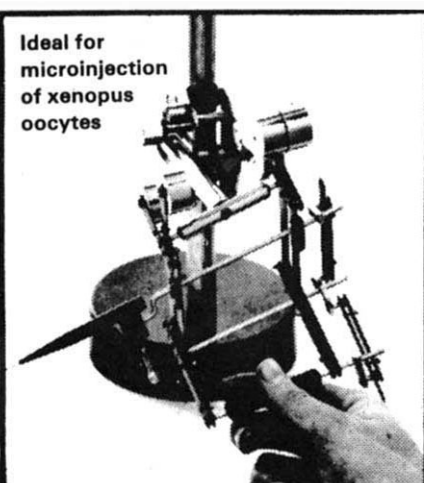
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● Perkin-Elmer has released the model R100 strip chart recorder for use with the company's instruments. The R100 is controlled by a microcomputer and features include digital pen movement. Furthermore, in addition to the usual time drive, a stepper motor chart drive allows operation in pulse drive to increase scan synchronization. Other features of the R100 include automatic return which allows the precise overlay of recordings; auto-advance so that the chart can automatically advance to the next grid line; 10 input ranges and 12 chart speeds; remote control and a non-slip chart. To prevent the chart from slipping, the recorder has a locking system so that the operator can write on the paper while it is recording or even tear it off without disturbing the pen. No mechanical clutches connect to the chart drive, so that backlash is reduced. *Circle No. 108 on Reader Service Card.*

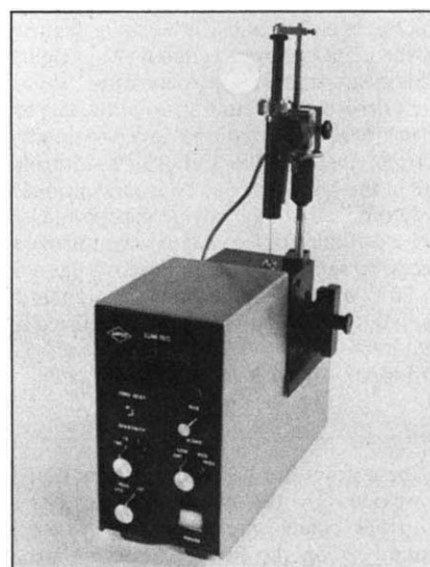
● Diamond Electro-Tech has added an instrument designed for intracellular and extracellular measurements to its line of neurophysiology equipment. The MPA-6 differential d.c. pre-amplifier is a high impedance, low-noise system. The MPA-6's differential field effect transistor input design isolates the unit from the ground, making it less susceptible to noise interference. The circuit has been designed to allow the high frequency cutoff to be reduced by connecting capacitors between the pins in the output connector. Diamond Electro-Tech has also produced the MPS-20 battery power supply, which powers the MPA-6. It may also be used with other instruments requiring a nominal supply voltage of $\pm 15\text{ V d.c.}$ The MPS-20 is recommended for powering neurophysiology instrumentation to eliminate the a.c. artifact, which may be encountered with outside power sources. *Circle No. 109 on Reader Service Card.*

● Lumi-Tec, a new luminescence detector with both peak and integration modes of digital analysis is now available from American Research Products. This instrument measures $11 \times 29\text{ cm}$ and has a reported sensitivity of 1.4×10^{-14} moles using a standard ATP reagent. Injection of sample or substrate may be manual, using a syringe, semi-automatic with a repeating dispenser, or automatic, using a flow-cell. Standard cuvettes supplied are $6 \times 50\text{ mm}$ with an optional $12 \times 47\text{ mm}$ size. The tube detector has a stability of $\pm 0.05\%$ full scale with dynamic range of 10,000 to 1. The Lumi-Tec has four ranges of sensitivity with three levels of blank subtract. The recorder outputs are 0-150 mV and 1 V full scale with a binary code decimal (BCD) option for printer/computer hook-up. Operation of the instrument is on 115 and 230 V, 50 to 60 Hz with 55 W of power. *Circle No. 110 on Reader Service Card.*

● A new heavy duty microtome is now available from Reichert-Jung. The Polycut microtome is designed for sectioning hard and large area specimens. It can handle samples up to $250 \times 200\text{ mm}$, and will cut sections ranging from 1 to $300\text{ }\mu\text{m}$. It is controlled by a separate electronic module which provides the choice of manual, semi-automatic and automatic operation of the motor drives. There is also provision for variable retraction on the return stroke and automatic changeover from pre-cutting to sectioning. The microtome has a universal clamping system such that bone and whole-brain sections can be prepared for sectioning. An ultramilling device is also available for work involving softer metals. *Circle No. 111 on Reader Service Card.*

● Sembly Medical has produced a brochure describing their SLP Neurostat, a new instrument for cryoanalgesia. The brochure lists ten sites to which effective cryoanalgesia can now be extended using the SL 2000 and its associated cryoprobes. *Circle No. 112 on Reader Service Card.*

● Diamond Electro-Tech has added four new units to its neurophysiology measurements line. These include a self-filling glass micropipette for intracellular measurements and three new electrodes for extracellular measurements. The extracellular electrodes have tips less than $1\text{ }\mu\text{m}$ in diameter and give a choice of electrical impedances. Diamond Electro-Tech offers both tungsten and stainless steel micro-electrodes. A third category of extracellular microelectrodes, a Paralene-C insulated tungsten microelectrode, is also available. Paralene-C is a bio-compatible polymer which is vacuum-deposited on the tungsten electrode substrate. It yields an electrode conformal coating that is free from pin holes. These electrodes are claimed to have a small tip exposure for a given impedance. *Circle No. 113 on Reader Service Card.*



Lumi-Tec from American Research Products

● A new instrument for looking at catecholamines has recently been introduced by BAS. The system, which can be used to determine levels of catecholamines and other neurochemicals, has dual electrode capabilities, which should decrease the time required for sample preparation. It should also enhance both the selectivity and the sensitivity of the assay. The analyser package supplied by BAS includes all the instruments, reagents and materials required for the analytical procedures.
Circle No. 114 on Reader Service Card.

● Medical Systems has expanded their Neuro Graph STA-1 system. The Neuro Graph STA-1 is a turn-key system which can be used in cellular electrophysiology to prepare interspike interval histograms, post stimulus time histograms, field potential averages and to perform data storage and printout functions. In the new model, data transfer, paging and peristimulus operations have been added.
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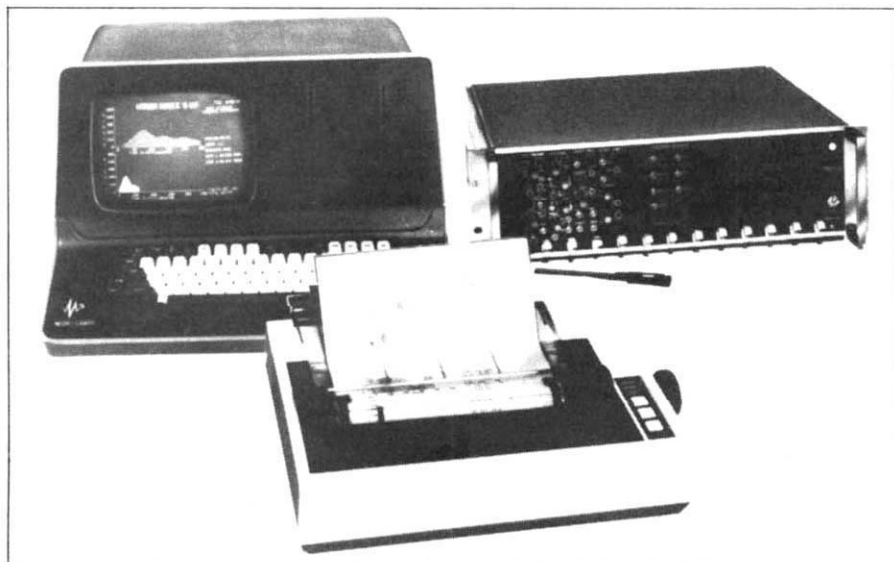
● A new system for use in the microiontophoretic and pneumatic ejection of drugs has been developed by Medical Systems. This new BH-2 system is rack mountable and is prepared to accept one balance module and up to five iontophoretic or pneumatic ejection modules in any combination. It will accommodate a 7-barrel micropipette. Push button controls activate the cycle, trigger, gate and continuous and termination functions.
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● Four procedures for the identification of cellular antigens by using antibodies directed specifically against them are illustrated and described on a new poster produced by Dako Corporation. The direct, indirect, peroxidase-anti-peroxidase and avidin-biotin methods are included and the recommended applications and limitations are listed.
Circle No. 117 on Reader Service Card.

● A new compact filter fluorometer, that features improved sensitivity and digital readout, has been introduced by Oriel. The model 450 fluorometer offers detection sensitivity greater than 100 pg with the most common fluorophores and has a wavelength range of 350 to 650 nm. This range can be extended to 750 nm with an optional photomultiplier. The optics are mounted in a block assembly to maintain alignment of the lamp, optics, sample and photomultiplier. Gain is switch-selectable and is calibrated for five orders of magnitude. The model 450 has fluorometric linearity typically better than 1%. Integrated circuit signal processing is used throughout and a strip chart recorder can be added.
Circle No. 118 on Reader Service Card.

● A copy of the Bakerbond application note on the separation of catecholamines by HPLC can now be obtained from J.T. Baker Research Products. The note includes full details of a rapid sample preparation procedure that uses the Baker 10 SPE system and shows the analytical conditions used and the chromatogram obtained with the Bakerbond aliphatic sulphonic HPLC column (4.6 × 250 mm). The chromatogram shows that dopa, noradrenaline, adrenaline and dopamine can be resolved within 10 minutes at a flow rate of 1 ml per minute.
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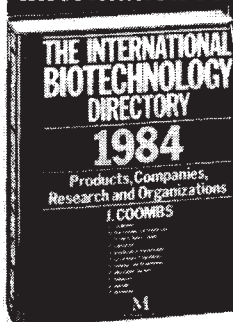
● A series of analysers for scanning transmission electron microscopy and transmission electron microscopy has been released by Edax (UK). The 9100 series includes an electron energy loss spectroscopy (EELS) option and a thin section analysis option. The EELS package includes software and hardware interfaces to connect EELS to the Edax analyser. The thin section option includes software to give data acquisition, a spectrum deconvolution and quantitative models for thin sectioned samples.
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Awards

The Royal Swedish Academy of Sciences has awarded the Crafoord Prize to **Professor Edward Lorenz** of the Massachusetts Institute of Technology and **Professor Henry Stommel** of the Woods Hole Oceanographic Institution for their contribution to understanding the large scale motions of the atmosphere and the sea. The prize consists of an award of £35,000.

The Charles Frederick Chandler Medal, presented by Columbia University for achievement in pure or applied chemistry, has been awarded to **Rudolph Marcus**, Arthur Amos Noyes professor of chemistry at the California Institute of Technology.

The Royal Society of Chemistry is offering a series of awards which have been financed by commercial companies. Twelve awards are being offered for 1983, each of which consists of a bronze medallion and £100. The fields covered include: analytical spectroscopy, chemical education, chemistry of the noble metals and their compounds, combustion chemistry, electroanalytical chemistry, enzyme chemistry, industrial analysis, macromolecules and polymers, medicinal chemistry, organometallic chemistry, synthetic organic chemistry and theoretical chemistry. Further information and application forms may be obtained from Dr John Gibson, The Royal Society of Chemistry, Burlington House, London W1V 0BN, UK and applications should be submitted before 30 November 1983.

The Lita Annenberg Hazen award committee is seeking nominations of physicians, or of a team of physicians, for the 1984 prize for excellence in clinical research. The award is given for research which constitutes a breakthrough in clinical research. The successful nominee will receive an award of US \$50,000 and an additional \$50,000 will be provided for the support of a research fellow or fellows whom the award winner will select as associates for up to three years. Further information is available from Dr Thomas Chalmers, Chairman of the Lita Annenberg Hazen Awards Committee, Mount Sinai School of Medicine, 1 Gustave Levy Place, New York 10029, USA.

Junior scientists wishing to be trained in epidemiology, biostatistics and environmental carcinogenesis — both chemical and viral — are invited to apply for training fellowships to the International Agency for Research on Cancer. Applicants should be engaged in research in medical or allied sciences and should intend to pursue a career in cancer research.

Fellowships are awarded for one year and are tenable at the agency or in another suitable institution abroad. In general, fellows will be selected from applicants with some postdoctoral research experience related to cancer in medicine or the natural sciences. They must have an adequate knowledge, both written and spoken, of the language of the country in which their fellowship is tenable. Stipends will vary according to the cost of living in the country of study, but the cost of the applicant's travel and, in certain circumstances, that of one dependent, will be met. Application forms and information are available from the Chairman of the Fellowships Selection Committee, International Agency for Research on Cancer, 150, cours Albert-Thomas, 69372 Lyon, Cedex 08, France. Applications must reach the agency before 31 December 1983.

The Fondation de Physiopathologie Professeur Lucien Dautrebande is to award a prize of 2 million Belgian francs in 1985 for work on human or animal clinical physiopathology which has therapeutic implications. Details about the prize may be obtained from the foundation at 35, chaussée de Liège, (5200) Huy, Belgium.

Appointments

Dr J. A. Catterall has been appointed secretary of the Science and Engineering Research Council, UK, in succession to Mr Brian Oakley.

Dr Burton Matthews has been appointed fourth president of the University of Guelph, Canada. His appointment will take effect on 1 January 1984.

Purnell Choppin and **Attallah Kappas**, both of the Rockefeller University, USA, have been appointed university vice presidents. **William Griesar** has been made vice president and general counsel of the university.

Professor T.S. West, director of the Macaulay Institute for Soil Research in Aberdeen, UK, has been appointed secretary general of the International Union of Pure and Applied Chemistry.

Professor John West is to remain as chairman of the British Council for Educational Technology (CET) for a further two years.

Meetings

1–2 December, **International Conference on Collective Phenomena**, Stockholm (Ms Joan Dale, 2 Frognaal Rise, London NW3, UK).

6 December, **Symposium on Advances in Inorganic Chemistry**, London (Mr Stanley Langer, The Royal Society of Chemistry, Burlington House, London W1V 0BN, UK).

6–8 December, **International Online Information Meeting**, London (Mr John Ozimek, Sales Promotion Executive, Learned Information, Besselsleigh Road, Abingdon, Oxford OX13 6LG, UK).

8–9 December, **Risk Management of Existing Chemicals**, Washington DC (Ms Shelley Kurtz, Chemical Manufacturers Association, 2,501 M Street NW, Washington DC 20037, USA).

19–20 December, **Meeting of the Inorganic Biochemistry Discussion Group of the Royal Society of Chemistry**, London, UK (Dr R. N. F. Thorneley, Hon. Secretary, Inorganic Biochemistry Discussion Group, Agricultural Research Council, Unit of Nitrogen Fixation, University of Sussex, Brighton BN1 9RQ, UK).

1–3 February, **The 1984 Sandoz Lectures in Gerontology**, Basle, Switzerland (The 1984 Lectures in Gerontology, Administrative Secretariat, PO Box 182, CH-4013, Basle, Switzerland).

5–7 February, **International Symposium on Auditory Biochemistry**, St Petersburg Beach, Florida (Dr Dennis Drescher, Chairman, 1984 International Symposium on Auditory Biochemistry, Department of Otolaryngology, Wayne State University, School of Medicine, Gordon H. Scott Hall of Basic Medical Sciences, 540 East Canfield Avenue, Detroit, Michigan 48201, USA).

17–19 February, **Astron — a Conference on the Science of the Sky and Subter — a Conference on the Science of the Earth**, Milan, Italy (Vincenzo Pagliuzzi, Comis Lombardia, Via Boccaccio 7, 20123 Milano, Italy).

19–22 February, **Joint Congresses in Recombinant DNA Research, Hybridoma Research and the Automation, Scale-up and Economics of Biological Process Engineering**, San Diego, California (Mr Randolph Charles, Director of Promotion and Public Relations, Scherago Associates, 1515 Broadway, New York 10036, USA).

11–16 March, **Annual Meeting of the American Society for Neurochemistry**, Portland, Oregon (Dr John Hammerstad, Local Organizing Committee Chair, Fifteenth Annual Meeting of the American Society for Neurochemistry, Department of Neurology, The Oregon Health Sciences University, 3181 SW Sam Jackson Park Road, Portland, Oregon 97201, USA).

19–31 March, **International Course on Cancer Epidemiology**, Rome (The Research Training and Liaison Unit, International Agency for Research on Cancer, 150, cours Albert Thomas, 69372 Lyon, Cedex 08, France).